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The effects of marginal and severe dietary magnesium and copper deficiencies on immune function

Windhauser, Marlene Marie Most, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1988

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THE EFFECTS OF MARGINAL AND SEVERE DIETARY MAGNESIUM AND COPPER DEFICIENCIES ON IMMUNE FUNCTION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences
(Physiology Option)

by
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B.S., Colorado State University, 1975
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December, 1988

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ABSTRACT

Throughout history, people speculated that an association existed between nutrition and disease prevention. Recently, studies have examined the influence of single nutrients on immunological mechanisms, but with limited focus on marginal deficiencies.

This project investigated the effects of severe, marginal, and adequate levels of dietary magnesium and copper on immune function. Three experiments were conducted, with 100 gm male rats consuming one treatment diet for three, four or eight weeks. Mineral levels used were: 1) magnesium - 50 ug/gm, 160 ug/gm, 280 ug/gm, 400 ug/gm; 2) copper - 0.5 ug/gm, 2.0 ug/gm, 3.5 ug/gm, 5.0 ug/gm; or 3) a combination of the severely deficient and marginal levels of magnesium and copper.

Results showed that within two weeks, plasma magnesium and copper levels reflected the amount of mineral in the diets. Ceruloplasmin paralleled plasma copper concentrations.

IgM and IgG concentrations correlated with plasma magnesium levels. Only the severely copper deficient rats had lower IgM. The gamma globulins were dramatically reduced with the combined magnesium and copper severe deficiency.

Lymphocyte proliferation following mitogen stimulation was not altered in magnesium deficient rats. Stimulation

indexes were lower with the severe copper deficient treatment. Proliferation rates after ConA stimulation for marginally copper deficient rats were higher, but the differences were not statistically significant. Antibody response to heterologous red blood cells tended to be lower in severely magnesium or copper deficient rats, and higher in marginally copper deficient rats.

The combined severe deficiency and combined marginal deficiencies with 2.0 ug copper/gm had higher proliferation rates with ConA and significantly lower rates with PWM. Mean specific antibody response tended to be lower in severely deficient rats.

Neutrophil function was not altered by magnesium or copper deficiencies. However, a greater percentage of neutrophils from severely magnesium and copper deficient rats ingested bacteria, but bactericidal activity was similar to neutrophils from control animals. Neutrophil myeloperoxidase activity was higher in rats with the combined severe deficiency.

The results from these experiments indicated a tendency toward altered immune function with severely deficient magnesium or copper diets, while marginal deficiencies did not always appear to have an effect.

Chapter 1

INTRODUCTION

In the beginning, man, as a hunter-gatherer, ate a variety of meats and plants. Through trial and error, he identified those foods that presumably resulted in good nutrition and resistance to diseases. Thus, it had been speculated by investigators, including Hippocrates, that the association of nutrition and disease prevention existed throughout history (Stinnett, 1983).

As man moved to farming and with the growth of cities, people became more dependent on single food sources. However, failure of crops and a limited availability of foods rendered the populations more susceptible to plagues throughout European history. The relationship between malnutrition and disease was becoming apparent.

Additional evidence of the association between malnutrition and disease could be found in the history of warfare. During early wars, when destroyed crops resulted in famine, the starving populations became victims to pestilence. That sequence continued through the Napoleonic Wars, the American Civil War, and World War I. Significant improvements in nutrition helped to control illnesses by the start of World War II.

While the information provided by famine and wartime experiences led to the hypothesis of an interaction between

nutritional intake and resistance to disease, much documentation was provided by field studies of malnourished individuals in developing countries. Widespread incidence of infectious disease and high infant mortality rates were common in those populations. It had been recognized that persons who were malnourished were more susceptible to disease (Gershwin, et al., 1985; Gross and Newberne, 1980; Stinnett, 1983).

Those field studies, along with clinical observations and experimental studies provided evidence in the 1960's for the direct and causal interrelationships between infection and malnutrition (Scrimshaw, et al., 1968). Two patterns of interactions were described: 1) malnutrition may alter host resistance to infectious disease, or 2) infectious disease may have a deleterious effect on the nutritional status of the host.

The primary target of early clinical and experimental studies of nutritional effects on immune mechanisms was during protein-calorie malnutrition (PCM). With the complexity of deficiencies present in general malnutrition, many physical and biochemical changes occur. Moreover, immunological functions are altered in many ways.

The size, weight, and cellular structure of the lymphoid organs are significantly altered by malnutrition (Gross and Newberne, 1980; Stinnett, 1983). Cell-mediated immunity, the humoral response, phagocytosis and the

complement system also were found to be affected by nutrition.

Cell-mediated immunity (CMI) is depressed during severe PCM (Gershwin, et al., 1985; Chandra, 1980; Stinnett, 1983). This also happens during mild or moderate malnutrition (McMurray, et al., 1981), and the depression of CMI appears to be in proportion to the severity of the malnutrition (Neumann, et al., 1975).

During PCM there is impaired differentiation of T-cell precursors with decreases in the number of circulating T-lymphocytes and an increased proportion of "null" cells. Lymphocyte response to mitogens and antigens generally is reduced, perhaps due to impairment in antigen processing or recognition. Cutaneous delayed hypersensitivity reactions also are diminished in malnourished individuals (Schlesinger and Stekel, 1974).

While alterations occur with T-lymphocytes in PCM, the number of circulating B-lymphocytes remains normal (Gershwin, et al., 1985; Gross and Newberne, 1980; Stinnett, 1983). However, the number may be increased at times when infections are present. Immunoglobulin levels usually are normal during PCM (Suskind, et al., 1976) or may be elevated with concurrent infections (Stiehm, 1980). Serum IgE significantly increases due to the prevalent incidence of intestinal parasitic infestations in malnourished individuals. Specific antibody responses have been found to vary depending on the severity of malnutrition, which

perhaps results from a lack of T-cell regulation (Neumann, et al., 1975).

Neutrophilia frequently is observed in malnourished individuals (Gershwin, et al., 1985; Gross and Newberne, 1980; Stinnett, 1983). But, variable results have been obtained and reported concerning the functions of the phagocytes during PCM. In general, chemotaxis and phagocytosis are normal, while the bacterial-killing capacity is impaired. Much of the variation might be explained by biochemical alterations of the phagocyte enzymes involved in the oxygen-dependent bactericidal system (Salimonu, et al., 1982).

The depression of immunocompetence that has been reported in malnourished individuals from developing countries similarly occurs in hospital patients in the United States (Dowd and Heatley, 1984). Nearly half of patients with chronic liver disease or chronic renal failure are affected with undernutrition and depression of cellular immunity. Undernourished surgical patients generally have increased incidence and severity of postoperative and terminal infections. Cancer patients are frequently undernourished, which results in decreased immunocompetence and a high risk to fatal infections. Nutritional supplementation has been beneficial in improving the immune function of malnourished hospitalized patients.

Alterations of the immune response in the elderly also may be ascribed to nutritional deficiencies (Chandra, et

al., 1982; Effros and Walford, 1987). Preliminary observations of malnourished individuals more than 60 years of age have shown impaired CMI with reduced T-cell numbers, decreased lymphocyte proliferation responses, and impaired delayed hypersensitivity skin reactions. Those immune responses improved after nutritional supplementation.

Dietary deficiencies also have been implicated in veterinary medicine as contributing to serious diseases in animals (Chandra and Newberne, 1977). Studies during the 1940's indicated that in animals, an adequate supply of protein discouraged intestinal parasites, and vitamin deficiencies decreased resistence to infection in varying degrees (Chandler, 1951).

In the 1960's, dietary deficiencies of California beef cattle were found to be closely related to high incidences of abortion caused by infectious bovine rhinotracheitis (Crane, 1965). Feed supplementation with phosphorus, vitamin A, protein and energy, in addition to vaccinations, increased the weaned calf crop from 73 to 94 percent.

Clinical observations of dogs in the 1960's found that malnutrition contributed to immunization problems (Sheffy, 1966). In malnourished dogs antibody production was lower than in healthy dogs after distemper and infectious canine hepatitis vaccination. Nutritional therapy with simultaneous immunization was found to facilitate a maximal immune response. Hospitalized dogs suffering from anorexia were found to be at greater risk to contracting infectious

diseases (Morris and Collins, 1967). The presence of anorexia was reported to affect drug therapy, wound repair or surgical intervention.

Food deprivation in small companion animals alters host-defense mechanisms much like PCM in humans (Lewis, et al., 1987). A reduction of T-lymphocytes depresses CMI responses, and humoral immunity also is impaired with low synthesis of antibodies. Leukocyte motility is decreased along with the bactericidal activity of the leukocytes. Likewise, similar changes in the immune response of small animals may occur with injury or disease when food intake is inadequate. Proper nutritional support is required for recovery and improved immunocompetence.

Today, the nutritional care of hospitalized companion animals receives attention to optimize recovery of critically ill or injured animals (Allen, 1986; Crowe, 1985). Supplemental diets or parenteral and enteral hyperalimentation methods are available to provide adequate nutrition for animals requiring nutritional support.

In addition to small companion animals, the nutritional status of livestock has been a concern (Miller, 1975). Modern feeding conditions of high animal density create favorable environments for the spread of infectious disease. Proper nutrition is now considered to reduce the incidence and severity of infections.

Overall, it has been shown that malnutrition impairs immunocompetence, and increases susceptibility to infection.

Thus, nutritional deficiencies such as those that occur with PCM can cause infections to become more severe. But this synergistic relationship between diet and infectious disease is bidirectional. Infections can have a detrimental influence on the nutritional state of an individual or animal through biochemical, metabolic, and hormonal responses (Beisel, 1977, 1980; Lewis, et al., 1987).

The nutritional consequences of infection are largely the result of catabolic events associated with fever and anorexia (Beisel, 1977, 1980). Changes occur in nitrogen and protein metabolism when amino acids are utilized for gluconeogenesis. The demand for amino acids also increases during the synthesis of proteins required for host defenses. Thus, negative nitrogen balance results. This is accompanied by altered production and utilization of carbohydrates and lipids. A depletion of body nutrient stores occurs during the hypermetabolic state of fever with changes in mineral and vitamin metabolism. Electrolyte imbalance occurs with water losses from diarrhea.

The studies showing the relationship of malnutrition and immunity involved multiple nutrient deficiencies. The many effects of PCM on immune function suggested that individual nutrients altered the immune response in some way. However, the causal relationship between single nutrients and immunologic functions could not be explained from previous studies. Therefore, the interrelationship of single nutrients and immunocompetence recently has received

more attention (Beisel, 1982; Beisel, et al., 1981; Chandra and Dayton, 1982; Nauss and Newberne, 1981).

Individual nutrient interactions with the immune system may be through direct or indirect mechanisms (Cunningham-Rundles, 1982; Gershwin, et al, 1985). Nutritional factors with primary activity within the lymphoid system may be classified as direct regulatory effects. Such effects may include those on cells of the immune system. The capacity of the cells to recognize foreign stimuli may be changed or antigen presentation may be modified. Phagocytic cells may become defective in the uptake or killing of organisms.

Nutrients required for other organ or cellular systems which then regulate the immune response interact indirectly. Metabolic, neurologic or endocrine alterations may ultimately influence immunologic mechanisms. For example, a deficiency of nutrients required for the synthesis of proteins may limit the production of immunoglobulins or lymphokines.

Various components of cellular and humoral immunity have been found to be altered by single nutrient deficiencies (Chandra, 1988). In general, protein and amino acid deficiencies reduce immunocompetence. Current research has shown that with protein malnutrition, functional activities of lymphokines and monokines are altered, and lymphocyte responsiveness is depressed.

Vitamin A deficiencies are associated with immunosuppression. Immunity also may be impaired with

deficiencies of the B vitamins which serve as cofactors in many metabolic pathways. Data on the roles of trace elements such as iron, zinc and copper, suggest the importance of those and other minerals in maintaining immunocompetence.

An animal's response to infections will be determined by its nutritional status (Chandra and Newberne, 1977). Animals consuming diets insufficient in the water-soluble vitamins generally have decreased resistance. Viral infections are more severe in animals consuming diets deficient in vitamin A. An inadequate protein intake impairs phagocytosis which depresses resistance to bacterial infections. Therefore, a balance of protein and energy is required for optimum immunocompetence, and proper nutrients must be provided for the animal to respond to the stress of a bacterial or viral challange.

The data obtained concerning single nutrient deficiencies with respect to immunocompetence may find practical application as more of those types of deficiencies are recognized. People on self-restricted diets or those receiving parenteral feedings may be deficient in a single nutrient (Beisel, et al., 1981). The elderly with limited incomes, low-birth-weight infants, or children with growth retardation may be "at risk." Single nutrient deficiencies also may occur in individuals or animals with chronic renal or liver disease.

In addition, marginal nutrient deficiencies are more common in industrialized nations rather than the severe

deficiencies that are associated with PCM (Fletcher, et al., 1988). This may be due to the consumption of highly refined and heavily processed foods that may have reduced contents of trace elements and/or vitamins. Marginal trace element status also may be found during periods of increased need for the nutrient such as growth and development, pregnancy, lactation, or during illness.

In the field of veterinary medicine, marginal or single nutrient deficiencies may occur in livestock consuming a limited variety of feeds, or primarily reared on home-grown forages and grains (Sheffy and Williams, 1982). The feeds may be low in a nutrient due to soil mineral deficiencies, or the nutrient may be lost during curing or storage. Severe environmental conditions or prolonged winter seasons may contribute to marginal total feed supplies.

Seasonal changes of some minerals in forages common to the Southeastern United States were found to be significant (Kappel, et al., 1985). Among those, magnesium and copper were found to be marginally deficient in relation to the requirements of lactating dairy cows at different times of the year.

Immunocompetence may be altered in various ways as a result of single nutrient or marginal deficiencies due to the multiple roles of nutrients and the complexity of the immune system itself. More information is needed about nutrient involvement in the immune system to provide a better understanding concerning the importance of nutrition

in host defense and resistance to disease. In addition, information concerning the physiological function of trace elements is so relatively new that little knowledge exists on the mechanisms by which trace elements affect the immune system (Gross and Newberne, 1980).

To contribute to the information concerning single nutrient and marginal nutrient deficiencies on immunocompetence, the two minerals, magnesium and copper, were selected for this study. The objectives were:

1. To determine the effects of adequate, marginal and deficient levels of dietary magnesium on immune function.

2. To determine the effects of adequate, marginal, and deficient levels of dietary copper on immune function.

3. To determine the effects on immune function when adequate, marginal, and deficient levels of dietary magnesium and copper are fed simultaneously.

The immune function was assessed by measuring activities of the cell-mediated and humoral immune systems, and phagocytic responses.

Plan of Development

In this chapter, the relationship between malnutrition and immunocompetence was discussed. The need for more research concerning single nutrient and marginal deficiencies was noted, and provided the basis for the three objectives and experiments of this research. The next chapter will summarize studies conducted on the effects of

magnesium and copper on immune function, while the third chapter will outline the procedures and analytic methods followed for each experiment. The results from each experiment will be provided in the fourth chapter. A summary of this research, a discussion for each experiment, and the conclusions will be presented in the fifth chapter.

Chapter 2

REVIEW OF LITERATURE

Early studies on the effects of nutrition on immunity looked at malnourished individuals and their lack of resistance to disease. Recently, individual nutrients have been examined as to their involvement in the immune system. Research on the two minerals selected for this study, magnesium and copper, will be reviewed.

Magnesium

Of the total body content of magnesium, 60 percent is in the bone, 1 percent is extracellular, while the remaining is distributed between muscle and other soft tissues. Magnesium is the second most abundant extra- and intracellular divalent ion. Magnesium homeostasis is maintained primarily by the kidney. A specific magnesium-regulating hormone has not been found. During magnesium deficiency, especially during youth, the bone provides a magnesium reserve and equilibrates the magnesium between the plasma and bone. In ruminants, however, the magnesium in the bone and soft tissue is not readily available. (Aikawa, 1981; Wester, 1987).

Most of the extracellular magnesium is ionized and biologically active, while approximately one-third is bound to serum proteins (Kroll and Elin, 1985). Of the protein-

bound magnesium, three-fourths is bound to albumin and the remaining one-fourth to the globulins. At high and low albumin concentrations, serum magnesium is linearly related to the albumin content. At the physiologic levels of albumin, magnesium concentration is independent of the albumin concentration.

Physiological Roles of Magnesium

During the process of biological evolution, magnesium was of primary importance for effective energy utilization. It was utilized for photosynthesis and then for oxidative phosphorylation. Magnesium is involved in metabolic processes including glucose utilization, muscle contraction and membrane transport systems (Aikawa, 1981; Wester, 1987).

The magnesium cation is complexed with many enzymes including those that catalyze the transfer of phosphate groups. Enzymes utilizing ATP have an absolute requirement for magnesium, with the magnesium ion bound to the phosphate moiety of ATP to form a stable complex. Thus, magnesium is involved in numerous reactions which are essential to life (Aikawa, 1981; Wester, 1987).

Magnesium also is important in all stages of protein metabolism (Aikawa, 1981). The concentration of intracellular magnesium must be optimal to maintain the structural integrity of the ribosomes for RNA synthesis. The transfer of amino acids in RNA translation requires magnesium, and peptide bond synthesis is dependent on

magnesium. During the synthesis of nucleic acids, magnesium, as well as ATP, are required. In addition, the DNA helix is stabilized by magnesium.

Magnesium Deficiencies

Magnesium deficiencies in man were largely unrecognized until recently. The importance of magnesium deficiency is associated with the major cardiovascular diseases, including hypertension, congenital heart failure and arrhythmias (Whang, 1987). Among the causes of clinical hypomagnesemia are gastrointestinal factors such as alcoholism, diarrhea, or malabsorption, renal losses with diuretics, or endocrine disorders including diabetes. Concurrent potassium depletion increases the risk of cardiac rhythm disturbances.

The daily magnesium intake may be suboptimal when compared to the U.S. Recommended Dietary Allowance (Wester, 1987). It has been reported that 25 percent of surveyed individuals had adequate dietary intakes. The refining of flour, rice, and sugar results in a loss of greater than 80 percent of the magnesium content. Boiling vegetables may diminish magnesium by half. Magnesium absorption may be decreased by excess calcium, phosphate, phytate, and protein. Lowered absorption increases the dietary requirement for magnesium.

In animals, magnesium was first demonstrated to be an essential nutrient in 1926. A deficiency in rats retards growth and causes peripheral vasodilation, anorexia,

hyperirritability, muscular incoordination, convulsions, coma, and death. Experimental animals that are deficient also have shown excessive calcification of bone, disturbances in calcium and phosphorus metabolism, depressed protein synthesis, and a high incidence of fetus resorption. Symptoms of magnesium deficiency in poultry include poor feathering, ataxia, and palpable tremors (Sell and Fontenot, 1980).

The National Research Council (1978) requirement of magnesium for the growing laboratory rat, based on an estimated feed intake of 15 gm/day, is 400 ug/gm. This amount also is adequate to maintain normal levels of blood and bone magnesium. The optimal level of magnesium for mice has not been established, but 400 ug/gm has been recommended. The American Institute of Nutrition's AIN-76 mouse diet includes 500 ug magnesium/gm diet.

The level of magnesium in common feed ingredients for most animals is high in relation to the dietary requirement. Therefore, deficiencies are uncommon, and are induced by experimental purified diets. Ruminants, however, are susceptible to hypomagnesemia or grass tetany, which is associated with a metabolic deficiency of magnesium. The deficiency may be the result of a simple dietary deficiency, or may be caused by an impairment of magnesium absorption or utilization by the animal (Sell and Fontenot, 1980).

Immunologic impairment due to magnesium deficiency has not been reported in humans. However, for many animals, the

immune system is affected by magnesium deficiencies (Beisel, 1982). It is believed that the roles of magnesium in DNA and protein synthesis influence the immune system. However, little is known about the specific biochemical interactions of magnesium in immunologic processes. More is known about the physiologic changes of the immune system in response to a magnesium deficiency.

Leukocytic Effects

Magnesium deficiencies in rats consuming diets with 30 to 60 ug magnesium/gm consistently produced leukocytosis and myeloid hyperplasia (Battifora, et al., 1968; Hass, et al., 1980). The increase in total leukocyte count was primarily due to a two- to threefold increase in the number of circulating neutrophils. Eosinophilia also was common in animals during the acute stage of deficiency. After two to three weeks, the leukocyte count declined. Feeding diets with adequate levels of magnesium, 650 ug/gm, rapidly returned leukocyte counts to normal in most rats. Extending the deficiency to 60 weeks by reducing the magnesium content of the diet, sustained the leukocytosis (McCreary, et al., 1967).

Battifora, et al. (1968) found with persistant and severe leukocytosis, myelogenous leukemia followed. Additionally, cells from leukemic rats were successively transplanted to newborn rats who then developed leukemia. In subsequent studies, malignant lymphomas appeared within 24

hours in younger rats fed magnesium deficient diets containing 30 to 50 ug magnesium/gm (McCreary, et al., 1973). When live lymphoma cells were injected into other rats, the deficient rats were more susceptible to transmission of lymphomas than normal control rats.

Normal and deficient rats then were immunized with sub-tumorigenic doses of viable lymphoma cells (Hass, et al., 1978). The rats were challenged with a tumorigenic dose of lymphoma cells, and 104 of 110 deficient rats died of lymphoma, while only 5 of 101 normal rats died. Two subgroups from the control rats then were subsequently challenged, after consuming a normal or a deficient diet for eight weeks. Normal rats survived the challenge. However, 90 percent of the newly deficient rats died of lymphoma indicating a loss of a previously established immunity to lymphoma.

Extensive mast cell degranulation, an increased release of histamine resulting in histaminuria, and hyperemic dermatitis have been found to occur with magnesium deficiency (Hass, et al., 1980). An abnormal distribution of mast cells with decreased granularity of the mast cells, but without increased urinary histamine also have been reported (Bois, 1963). Peripheral vasodilation was observed after 10 days of magnesium deficiency and peripheral hyperemia within 20 days. The hyperemia was replaced by white edema with chronic magnesium deficiency lasting 60 days.

The changes in mast cell activity and histamine levels were further studied in magnesium deficient rats (Kraeuter and Schwartz, 1980). Widespread degranulation of the mast cells occurred early during magnesium depletion with a 50 ug magnesium/gm diet. Subsequently, degranulation subsided, but the number of submucosal mast cells increased and were smaller in size than those from control rats consuming 1,000 ug magnesium/gm.

Blood histamine levels in the magnesium deficient rats peaked within 12 to 16 days which correlated with maximal degranulation. Although submucosal mast cells increased later, blood histamine levels declined. Peritoneal mast cells contained significantly more histamine within five days of magnesium deficiency, then dropped to one-third the amount in cells collected from control rats. The authors concluded that the immature cells at later stages of magnesium depletion were deficient in their capacity to secrete histamine.

Lymphoid Organs

Thymic hyperplasia has been observed in rats during magnesium deficiency (Alcock, et al., 1973). In various experiments, rats consumed diets with a magnesium content of 10 ug/gm. Enlarged thymuses were observed between the 38th and 60th day of deficiency in 18 to 52 percent of the deficient rats. The incidence of enlarged thymuses was twice as great in young rats than among older animals. The normal

cellular structure of the thymus was replaced by cells that resembled transformed lymphocytes. The change was hyperplastic rather than neoplastic. Splenomegaly also was observed in the deficient rats.

In an attempt to help explain the changes occurring in the lymphoid organs during magnesium deficiency, the synthesis of tissue proteins and nucleic acids was measured (Zieve, et al., 1977). Rats were fed a diet containing 50 ug magnesium/gm for 4.5 months before isotope incorporation studies were conducted. The rats were injected intraperitoneally with [^{3}H]leucine for protein incorporation, [^{3}H]thymidine for DNA incorporation, or [^{14}C]orotic acid for RNA incorporation. The average increase in thymic weight was not significant, but the spleens were twice the weight of those from pair-fed control rats and were congested with lymphocytes.

Uptake of [^{14}C]orotic acid in the spleen and thymus significantly increased, but its incorporation into RNA did not change. [^{3}H]Thymidine incorporation into splenic and thymic DNA dramatically increased. The increase in DNA synthesis was believed to represent early lymphoproliferative processes leading to leukocytosis and the development of neoplasia. On the contrary, protein synthesis appeared to be inhibited in magnesium deficient rats, because [^{3}H]leucine incorporation was significantly lowered in the spleen and thymus. The reduction in protein

synthesis may have been responsible for impaired immune response during magnesium deficiency.

Immunoglobulins

With the changes that occur in the lymphoid organs during magnesium depletion, and with the requirement of magnesium for protein synthesis, the effects of magnesium deficiencies on immunoglobulin synthesis and secretion have been of interest (Alcock and Shils, 1974; Elin, 1975; McCoy and Kenney, 1975). Rats fed a magnesium deficient diet for two, four or six weeks had lower levels of serum IgG than animals fed a control diet. Animals repleted with injections of magnesium and fed adequate diets showed after 24 hours an increase of serum IgG. IgG levels then were similar to or greater than control values. The marked depression and rapid recovery of serum IgG in response to magnesium intake suggested a direct role for the mineral in the synthesis or metabolism of the immunoglobulin (Alcock and Shils, 1974).

Elin (1975) evaluated four serum immunoglobulin concentrations in mice fed 35 ug magnesium/gm. Levels of IgG_1, IgG_2, IgA, and IgM significantly decreased after six days of the dietary deficiency. After 12 days, the IgG_2 and IgA concentrations increased, and the IgG_1 concentration was near control values. The partial recoveries were explained by protein catabolism which might have provided endogenous magnesium that could have been reutilized.

The mice also were immunized with sheep red blood cells, and after four days the number of splenic plaque-forming cells (PFC) were determined. The spleens from magnesium depleted mice were similar in weight to those from control mice, but the deficient mice were unable to gain total body weight. Although splenic weights were not significantly different, magnesium deficient mice had a significant decrease in the total number of PFCs per spleen.

The splenocytes also demonstrated a reduced ability to respond to an antigenic stimulus and synthesize IgM specific for the antigen, as indicated by lowered serum IgM concentrations. Therefore, the process of antigen recognition and processing with subsequent antibody synthesis was impeded with magnesium deficiency.

In an analysis of the production of specific antibody, serum gamma globulin levels of young and older magnesium deficient rats were measured, in addition to agglutinin and hemolysin activities (McCoy and Kenney, 1975). Weanling rats were fed a diet with 10 ug magnesium/gm for eight days and then 142 ug magnesium/gm. The rats were immunized after 36 days with sheep red blood cells. Older, 200 gram rats were fed 10 ug magnesium/gm, and immunized after 29 days.

Spleens, when expressed as percentages of body weights, were heavier in the magnesium deficient young and older rats than in control rats. The young magnesium deficient rats had higher concentrations of gamma globulins than controls, but agglutinin and hemolysin titers were lower. Older deficient

rats had lowered concentrations of gamma globulin in addition to lower agglutinin and hemolysin titers.

The low antibody titers associated with magnesium deficiency might have occurred due to a failure to synthesize antibody. This was reflected in the older animals that had lower concentrations of gamma globulins. However, the effect of magnesium was believed to be primarily on reduced activity of specific antibodies, especially in younger animals. The authors believed the lack of dietary magnesium influenced cellular metabolism in such a way as to depress serum antibody titers.

In contrast to the general decrease seen in the production of most immunoglobulins, the level of circulating IgE increases (Larvor, 1980). Three- to fourfold increases in serum IgE have been observed in experiments with magnesium deficient mice. Several factors were thought to be responsible for the increase. Usually, increased IgG inhibits IgE production. Therefore, the decrease in IgG levels during magnesium deficiency may stimulate IgE production. In addition, thymic alterations in magnesium deficiency may decrease the control of IgE synthesis.

Recently, the effect of magnesium on the <u>in vitro</u> binding of IgE by human basophils has been studied (Pruzansky and Patterson, 1988). Optimal binding of [^{125}I]IgE to basophils occurred when suspended in medium without Mg^{2+} and Ca^{2+}. Binding to basophils incubated in medium containing Mg^{2+} and Ca^{2+} was significantly reduced.

The cations seemed to decrease the number of membrane-bound receptors available for IgE binding. When 10 percent fetal calf serum was added to the leukocytes, binding significantly increased. The authors suggested that a Ca^{2+} and Mg^{2+} energy-requiring process shed basophil membrane-bound IgE receptors, and factors present in fetal calf serum were response for reexpression of the receptors.

Lymphocyte Proliferation

The process of antibody formation includes the transformation of lymphocytes to lymphoblasts. Therefore, the effects of magnesium deficiencies on lymphocyte stimulation have been studied (Gunther and Averdunk, 1979). Spleen and thymus cells from chronically magnesium deficient rats had [^{3}H]thymidine incorporation rates which were half the control values. DNA and protein contents of the cells were 25 percent lower than in cells from normal rats. When stimulated by phytohemagglutinin (PHA), lipopolysaccharide (LPS), or concanavalin A (Con A), the cells from normal rats had response rates that were 1.5 times higher than the cells from the magnesium deficient rats.

The spleen and thymus cells also were incubated in medium with a low magnesium concentration. The DNA synthesis rates and the responsiveness to lectins were lower for cells from both the magnesium deficient rats and the normal rats when compared to cells incubated in medium with a higher magnesium content. The authors proposed that the magnesium

deficiency altered cell permeability causing changes in electrolyte concentrations. Increased intracellular sodium and calcium would inhibit DNA and protein synthesis, decreasing cell proliferation.

In a series of experiments on the effects of magnesium on DNA synthesis, depletion of Mg^{2+} in growth medium decreased DNA synthesis in chicken embryo fibroblasts and mouse BALB/c 3T3 cells (Sanui and Rubin, 1982). Cellular cation content changed with a dramatic increase in intracellular calcium content. The effects of reduced extracellular magnesium on cell membrane permeability and the cellular Ca^{2+} pump appeared to be responsible for the reduction in DNA synthesis.

After measuring levels of intracellular magnesium in mouse BALB/c 3T3 cells, Sanui and Rubin (1982) found a positive correlation between intracellular Mg^{2+} levels and DNA synthesis. As the rate of [^{3}H]thymidine incorporation increased, intracellular magnesium had an overall increase of 15 percent. In addition, a slight change in intracellular magnesium corresponded with a substantial change in the incorporation rate of [^{3}H]thymidine. It therefore was suggested that intracellular magnesium was primary to the physiological regulation of cell metabolism and growth.

A study to quantify lymphocyte proliferation requirements for magnesium, in addition to calcium, employed a serum-free <u>in vitro</u> culture system (Abboud, et al., 1985). DNA synthesis in lymphocytes from healthy human donors was

measured by the incorporation of [^{3}H]thymidine into the lymphocytes after exposure to phytohemagglutinin (PHA) and concanavalin A (Con A). The cells were suspended in a serum-free modified Dulbecco's medium without added calcium and magnesium. The inherent calcium concentration was 25 uM and the magnesium concentration was 7 uM. Magnesium and calcium then were added at concentrations ranging from 0 to 1 mM.

The addition of either 1 mM magnesium or 1 mM calcium increased thymidine incorporation into DNA in the presence of PHA or Con A. When both magnesium and calcium were added to the medium, thymidine incorporation greatly increased, indicating a synergistic effect. In medium that contained 1 mM calcium, maximal DNA synthesis occurred with the addition of magnesium, while 70 percent of the maximal response was observed without added magnesium.

The authors concluded that the lower requirement for external magnesium than for calcium may be due to the manner in which the lymphocytes maintain the concentrations of the cations. Intracellular magnesium concentrations are similar to external concentrations, while cytoplasmic calcium is actively removed. Magnesium-dependent enzymes for lymphocyte DNA synthesis may have a greater retention of magnesium when the external concentration is low.

Results from experiments on the role of magnesium ions in the proliferation and differentiation of human promyelocytic leukemia HL-60 cells also confirmed the requirement of magnesium (Okazaki, et al., 1987). HL-60 cell

proliferation peaked in standard medium containing 0.4 mM magnesium, while in magnesium deficient medium, proliferation was inhibited. After two days, cells were resuspended in standard medium, and cell proliferation was restored.

Cell differentiation was induced by the addition of 1 alpha, 25 dihydroxyvitamin D_3. Maximum differentiation was observed in cells incubated in standard medium. For cells incubated in magnesium deficient medium, differentiation was inhibited, but when transferred to the standard medium after two days, the cells differentiated.

In contrast, cells from standard medium that were resuspended after two days in a magnesium deficient medium, showed no further increase in differentiation. The authors suggested that the expression of differentiation-related phenotypes required magnesium, but magnesium was not required for the commitment to differentiate. Additional experiments found that the mechanism inhibited by magnesium deprivation was during protein synthesis. A reduction of extracellular magnesium decreased intracellular magnesium to 61 percent of the control values after three days, and protein synthesis was decreased.

Neutrophil Function

The production and function of polymorphonuclear neutrophils (PMN) require extracellular magnesium (Brennan, et al., 1980). The neutrophil arises from a colony forming

unit precursor that requires magnesium and calcium for optimum proliferation. Depletion of either cation in cultured cells, resulted in reduced proliferation, and the absence of both severely restricted colony growth. However, in vivo deficiencies produce neutrophilic leukocytosis, suggesting that sufficient cation content may exist to release leukocyte production from inhibition.

Neutrophil adhesion and aggregation requires magnesium, but not calcium. Extracellular magnesium also is needed for chemotaxis and spontaneous movement of the neutrophil. Optimum chemotaxis was observed when calcium was present, although either cation supported chemotaxis. Magnesium, however, was more potent.

Similar results have been observed for phagocytosis (Stossel, 1973). Optimum ingestion by human neutrophils of serum-opsonized oil particles was observed when magnesium and calcium were present in the incubation medium. When unopsonized particles were used, higher concentrations of magnesium or calcium were required for ingestion. In both situations, magnesium was more effective than calcium.

During phagocytic ingestion of microorganisms, oxygen is consumed and rapidly converted to the superoxide anion, hydrogen peroxide and the hydroxyl radical, which are used for microbicidal activity. The enzyme system involved in the respiratory burst is NADPH oxidase. The role of magnesium and calcium ions in NADPH oxidase activity have been examined (Johnston, et al., 1985).

Kinetic studies of the NADPH oxidase from membrane preparations of human neutrophils were performed with the addition of various cations in the reaction mixture. The production of the superoxide anion was enhanced by either Mg^{2+} or Ca^{2+}. EDTA inhibited NADPH oxidase activity, but was overcome by the addition of excess Mg^{2+} or Ca^{2+}. Saturation studies suggested that the magnesium ion was required to convert the enzyme to its active form, but had no effect on the binding of the substrate.

In a discussion of the results, the authors considered possible mechanisms of the magnesium ions in NADPH oxidase activity (Suzuki, el al., 1985). The membrane fraction might have contained other components that modulated the catalytic activity. Binding of the Mg^{2+} with catalytic or regulatory components of the oxidase might have enhanced oxidase activity.

Copper

Total body copper content exists primarily in the liver, heart, kidneys, spleen, and brain (Davis and Mertz, 1987). The distribution varies according to age, copper status, and species of animal. Of the copper present in blood, half is found in the erythrocytes with smaller amounts in the white blood cells and platelets. Sixty percent of the erythrocyte copper is complexed with the enzyme, superoxide dismutase (SOD), while the remaining exists as a labile pool complexed with amino acids.

In the plasma, 90 to 93 percent of the copper is firmly bound to ceruloplasmin (Cp). The remaining is less firmly bound with albumin (Davis and Mertz, 1987). In rats, less copper is bound to Cp, and in dogs, 30 to 35 percent of serum copper is bound with proteins and amino acids (Brewer, 1987).

Copper homeostasis is primarily maintained by the liver which is the principle storage organ for copper (Suttle, 1987). Plasma and tissue enzymes also comprise the copper pool. When copper intake is inadequate, species variations exist. Rats first increase the amount of copper absorbed from the diet, which then is retained in the liver. Ruminants typically have a high capacity for hepatic storage so, from the beginning, will mobilize copper from the reserves.

Physiological Roles of Copper

Copper is a multifunctional element, and is involved in many metabolic pathways (Suttle, 1987). Much of its involvement is related to its activity in metalloenzymes. Copper is required for heme synthesis through the iron mobilization activity of the enzyme Cp. During copper deficiency, anemia develops and iron accumulates in the liver. The structural integrity of connective tissue and the cardiovascular system is dependent on copper. The formation of elastin and collagen cross linkages is catalyzed by the copper-containing enzyme, lysyl oxidase. The mineralization

of collagen for bone growth also is dependent on copper, as well as the myelination of the central nervous system.

A reduction in plasma copper follows a fall in plasma Cp (Frieden and Hsieh, 1976). The multifunctional enzyme is involved in iron mobilization from storage sites to the plasma through its ferroxidase activity. Characteristic signs of copper deficiency in rats include anemia and hypoceruloplasminemia (Prohaska, et al., 1983). The copper transport function of Cp had been doubted, but there is evidence that Cp provides copper for copper-dependent enzymes, including cytochrome C oxidase, lysyl oxidase, and hepatic SOD (Camakaris, 1987). Cp does not appear to be required for copper uptake by lymphocytes, but further evaluations are required.

The relationship between dietary copper and another copper-containing enzyme, SOD, has been investigated within the last 15 years. Changes in erythrocyte SOD activity have been compared with changes in the copper status of various animals. Reduced SOD activity was demonstrated with a decrease in dietary intake of copper in swine (Williams, et al., 1975), cattle (Paynter and Allen, 1981) and lambs (Andrewartha and Caple, 1980). When the deficient lambs were fed diets adequate in copper, SOD activity increased within 90 days.

Similar results were reported by Suttle (1983) in steers, lambs, and ewes. During dietary copper depletion, a decline in SOD activity began later and was slower than the

decline in plasma copper. SOD activity began to increase after plasma copper repletion, and continued more slowly.

The magnitude of change in SOD activity was found to be tissue specific in the rat (Paynter, et al., 1979) and in mice (Prohaska, 1983; Prohaska, et al., 1983). Liver SOD activity was greatly reduced during dietary copper deficiency, and was repleted with copper supplementation. SOD activities of spleen and thymus tissues also were lowered with copper deficient diets.

The significance of SOD in immunocompetence is thought to be related to its function as a scavenger of superoxide which is formed and released by phagocytosing neutrophils. Superoxide damages cells unless dismutated to hydrogen peroxide and O_2. SOD catalyzes this reaction, protecting the phagocytes and surrounding tissues (Fridovich, 1983).

Copper Deficiencies

Copper deficiency is rare in humans (Turnlund, 1988). Hypocupremia was first observed in the 1950's and was reported in 1964. Most deficiencies have been noted in patients on long-term total parenteral nutrition without copper or in premature infants fed cow's milk. Low serum copper and ceruloplasmin due to defects in copper metabolism are found in individuals with Menke's kinky hair syndrome or Wilson's disease. Most diets generally adequate in other nutrients contain sufficient copper. There is no RDA for copper, but a recommended intake range has been established.

On the other hand, copper deficiency is common in livestock (Brewer, 1987). It may be due to genetic factors, low copper content of feed or water, an excess of antagonistic minerals, or ingestion of some plant poisons. Food sources have great variations in copper content according to soil conditions, seasonal variations, weather, and processing procedures (Davis and Mertz, 1987).

Calcium, iron and zinc are antagonistic to copper absorption. Ascorbic acid depresses the bioavailability of copper through the reduction of the copper ion, or by the formation of stable complexes, similar to those that are formed with phytates. Minimum copper requirements exist depending on the presence of influencing factors. A greater understanding of the etiology of copper deficiency in grazing ruminants recently has led to improved diagnosis of hypocuprosis (Suttle, 1986).

In 1928 it was recognized that copper, in addition to iron, was required to alleviate the anemia which developed in rats on a milk diet. Since that time, the involvement of copper in a variety of metabolic processes became evident, but many of the biochemical functions of copper associated with specific deficiency symptoms are not completely understood (Davis and Mertz, 1987).

The satisfactory dietary copper content for the laboratory rat has been set at 5 ug/gm by the National Research Council (1978). This amount has been established for adequate growth and for the maintenance of maximal

hemoglobin concentration with a recommended iron allowance of 35 ug/gm. Together, the levels of copper and iron prevent hypochromic microcytic anemia.

No studies on the copper requirement of the mouse have been reported, but mouse requirements have been assumed to be similar to the rat (National Research Council, 1978). Adequate purified diets contain at least 4.5 ug copper/gm, and the AIN-76 mouse diet contains 5 ug copper/gm.

Characteristics of copper deficiency include anemia, neonatal ataxia, skeletal abnormalities, achromotrichia, impaired keratinization, cardiovascular disorders, and reproductive failure. Nutritional copper deficiency also may adversely affect the immune system (Davis and Mertz, 1987). The physiological and biochemical processes responsible for reduced immunocompetence caused by a lack of copper currently are being examined.

Bacterial Challenge

Several studies have reported a decreased resistance of copper deficient experimental animals to bacterial infections. Newberne and coworkers (Newberne, et al., 1968; Newberne, 1977; Gross and Newberne, 1980; Nauss and Newberne, 1981) found that copper deficiency resulted in high mortality of rats infected with Salmonella typhimurium. There was a significant reduction in both the average time of survival and the number of survivors of infected copper depleted rats when compared to infected control rats.

The spleens of the copper deficient animals failed to enlarge in response to the infection, unlike those from infected control rats. The livers also showed no tissue hyperplasia. In addition, total serum protein levels were lower, there was no increase in the serum gamma globulin content, and lymphocytes of copper deficient rats exhibited less mitogenic stimulation than those of supplemented controls. Because of these results, it was hypothesized that copper deficiency in rats prevented the reticuloendothelial system (RES) from responding appropriately to infection.

Jones and Suttle (1983) also found a higher incidence of mortality in subclinically copper deficient mice following intraperitoneal injection of Pasteurella haemolytica. Mice were fed diets with less than 0.001 ug copper/gm for 8 or 12 weeks before bacterial challenge. No clinical signs of copper deficiency were observed. Total body weight, spleen and liver weights, and whole blood hemoglobin concentrations did not significantly differ from control animals. However, plasma copper decreased 68 percent and blood cell SOD activity decreased 26 percent.

Copper deficient mice that survived bacterial challenge had significantly enlarged spleens with a concomitant drop in body temperature when compared to uninfected animals. Several possibilities for the enhanced susceptibility of copper deficient mice to bacterial infection were discussed. Adverse effects on the RES were disputed due to a discrepancy in the results concerning enhanced splenic

response after challenge with the reported failure of response by Newberne et al. (see above). Impaired lymphocyte proliferation, T- and B-cell cooperation, and/or antibody production were suggested as possible explanations for the decreased resistance to infection.

Lymphoid Organs

The biochemical and morphological features of the spleen and thymus were found to be altered by chronic dietary copper deficiency (Prohaska, et al., 1983). Thymus glands were small in 6-week-old mice fed diets containing 0.6 ug copper/gm, while spleens were enlarged. Lymphoid tissue morphology showed abnormal mitochondria and misshapen nuclei. The copper deficient mice were anemic and had hypoceruloplasminemia. Biochemical changes observed were diminished activities of the cuproenzymes cytochrome oxidase and SOD in liver, spleen, and thymus tissues. These effects of copper deficiency on lymphoid tissue were suggested as responsible for impaired immunocompetence.

Antibody Response

Prohaska and Lukasewycz (1981) found humoral-mediated immunity to be impaired in copper deficient mice. Young mice were fed diets containing 0.5 ug copper/gm for four to six weeks. Response to the diets was variable so the mice were divided into two groups based on ceruloplasmin activity. Severely deficient mice had a 97 percent or greater decrease

in Cp, while those with a smaller decrease (69 percent) were considered marginally deficient.

Body weight, hemoglobin, liver copper, and antibody-producing cells significantly decreased in both groups of mice, with the largest changes seen in the severely deficient mice. Spleen weight increased only in mice considered severely deficient.

Serum ceruloplasmin activity was highly correlated with the number of antibody-producing cells, assayed in splenocytes after the mice were injected with sheep red blood cells. Also, the reduction in hemoglobin and liver copper followed the magnitude of immune response. Thus, the degree of immune function impairment was related to the severity of copper deficiency. The results suggested that a clinically marginal copper deficiency would impair the humoral-mediated response (Lukasewycz and Prohaska, 1981).

Antibody production, natural killer (NK) cell cytotoxicity, and delayed-type hypersensitivity (DTH) responses were assessed in copper deficient rats (Koller, et al., 1987). Rats were fed copper depleted (0 ug/gm), copper deficient (2 ug/gm) or copper adequate (6 ug/gm) diets for eight weeks. At six weeks, the rats were immunized and boosted eight days later with keyhole limpet hemocyanin (KLH).

Reductions in the DTH reactions were observed in rats consuming the copper depleted diets, but the changes were not significant. Rats in that group had a significant

decrease in NK cytotoxic activity, while rats on the 2 ug/gm diet had responses similar to control animals.

Antibody titers to KLH were ranked, and comparisons were made on the mean ranks for males and females on each diet. Male rats from the 0 ug/gm and 2 ug/gm diet groups, and female rats from the 0 ug/gm diet group, had reduced antibody responses. The decreases in antibody production and NK cytotoxicity were postulated to be caused by changes in copper dependent enzymes.

Lymphocyte Proliferation

Previous studies found copper deficient animals had lower resistance to infection. This in turn, led to speculations that cell-mediated immunity may be altered in some way. Lukasewycz and Prohaska (1983) determined the effects of dietary copper deficiencies in mice on T- and B-cell reactivity to mitogens. Splenocytes from mice maintained on a 0.6 ug copper/gm diet were stimulated with Con A, LPS, PHA, and pokeweed mitogen (PWM).

Stimulation indexes were variable, but the responses of the splenocytes from the copper deficient mice were consistently lower than those from adequate control mice. The mean stimulation indexes were less than 50 percent of the controls. Additionally, the decrease in responsiveness appeared to follow the decrease in ceruloplasmin activity.

Additional studies to evaluate the functional responsiveness of splenocytes from copper deficient mice

recently were conducted by Lukasewycz, et al. (1987). Two experimental designs were used to compare mixed lymphocyte reactions of splenocytes from copper deficient and control mice. Two strains of mice, C58 and C57BL, were used. First, splenocytes were stimulated by mitomycin-treated spleen cells from normal mice. Second, splenocytes from normal mice were stimulated by copper deficient or copper adequate mitomycin-treated spleen cells.

For the first experiment, the mean response of the splenocytes from copper deficient C58 mice was 49 percent of that for control mice. Similar trends were observed in C57BL mice, but mean comparisons were not significant. The copper deficient C57BL mice had hematocrits similar to controls so were considered marginally deficient. In the second set of experiments, splenocytes from the copper deficient mice of both strains stimulated less than splenocytes from control mice.

The reduction of immunocompetence appeared to be proportional to the degree of copper deficiency. Moreover, it was postulated that the deficiency altered lymphocyte membranes in such a way as to change the components necessary for stimulating mixed lymphocyte reactions.

Reduced lymphocyte proliferation during copper deficiency was found to differ according to tissue source of the lymphoid cells (Davis, et al., 1987). Spleen lymphoid cells and cervical lymph node cells from rats consuming copper deficient diets (0.6 ug/gm) for 14 to 70 days were

stimulated with Con A. Proliferation rates were influenced by the dietary deficiency. Spleen cells from the deficient rats had significantly lower proliferation rates than those from rats consuming adequate diets. In contrast, the proliferation of cervical lymph node cells from deficient rats was higher than controls.

Additionally, proliferation rates of non-adherent spleen cells were found to be equivalent to those from the spleen lymphoid cells of deficient and control rats. The phagocytic activity of spleen lymphoid cells from the deficient rats was lower than those from controls, while no differences were found for non-adherent spleen cells. The authors suggested that copper deficiency may reduce lymphocyte proliferation due to an impaired macrophage enhancer function for lymphocyte proliferation.

Neutrophil Function

The increased susceptibility to infection during copper deficiency had raised questions as to whether phagocytic activity was altered, especially through the copper-containing enzyme, SOD. The relationship between plasma copper concentrations, leukocyte SOD and phagocytic function has been investigated (Jones and Suttle, 1981). Peripheral blood leukocytes from copper deficient ewes, lambs and calves demonstrated unimpaired phagocytic activity of Candida albicans in vitro. On the other hand, the killing capacity of the leukocytes from copper deficient ruminants

was significantly reduced when compared to those from control animals. Copper repletion of calves restored candidacidal activity.

The SOD activities in erythrocytes and leukocytes from copper deficient ewes were significantly less than those from control animals. The decrease in SOD activity in erthrocytes was much greater than the decrease in leukocyte SOD activity. In addition, leukocytes from copper deficient ewes tended to produce more superoxide anion after stimulation, but the results were variable. The authors suggested that during copper deficiency, the decrease in leukocyte SOD and increase in extracellular O_2^- enhanced oxidative damage, and thus, impaired cell function and candidacidal activity.

The involvement of copper in cellular SOD anti-oxidant systems prompted an investigation of infection and neutrophil candidacidal activities during copper deficiency (Arthur, et al., 1981; Boyne and Arthur, 1981; Arthur and Boyne, 1985). Neutrophils of copper deficient cattle were able to ingest C. albicans, but killing of the yeast cells was impaired. Unlike other studies, the activity of SOD in neutrophils of the copper deficient cattle was found to be similar to that in neutrophils from animals receiving a copper-supplemented diet. Moreover, the concentration of O_2^- generated in stimulated neutrophils from copper deficient cattle was normal.

Therefore, the copper deficiency did not appear to cause the decreased killing activity by means of superoxide damage to the neutrophils. Bovine neutrophil SOD activity was not sensitive to changes in copper status of the animal. It was suggested that instead, the mechanisms of superoxide production were inhibited in copper deficient animals to affect candidacidal activity.

Later, Boyne and Arthur (1986) induced copper deficiences in cattle with elevated concentrations of molybdenum or iron in the diet, and investigated neutrophil function. Phagocytosis of C. albicans was impaired in neutrophils from cattle made copper deficient by supplemental molybdenum or iron. Candidacidal activity also was decreased. The results from this study might have been due to a more severe copper deficiency than in previous studies, or due to effects of the molybdenum or iron.

Summary

In conclusion, deficiencies of magnesium or copper have been found to alter immune function including the cellular and humoral systems. Magnesium deficiencies appear to alter the immune system through the mineral's involvement in protein and DNA synthesis. Antibody titers are low and lymphocyte proliferation is decreased. The effects observed during copper deficiency may be caused by changes in the activities of cuproenzymes. In particular, the decrease observed in phagocytic killing of microorganisms is thought

to be influenced by altered enzyme mechanisms during microbicidal activities.

Most of the studies, however, involved severe dietary deficiencies of magnesium or copper. Magnesium deficiencies in the rat and in mice have been induced with diets containing 10 to 60 ug magnesium/gm, or 2.5 to 15 percent of the NRC requirement. In one study, the magnesium content was increased from 10 ug/gm to 142 ug/gm, or 35 percent of the requirement to improve the physical condition of the rats for sampling.

Many of the in vitro studies used leukocytes from normal subjects, and changed the Mg^{2+} ion concentration of the incubation media. Few have used leukocytes from magnesium deficient animals.

Copper deficient diets have contained less than 0.6 ug copper/gm or 12 percent of the NRC requirement for the rat. One study included a marginal level of 2 ug/gm (40 percent of the requirement). The deficient diets for mice also contained less than 0.6 ug copper/gm, which also is 12 percent of the NRC recommended level for the laboratory mouse.

A few studies considered the experimental animals to be marginally copper deficient even after consuming a copper depleted diet. This was based on the fact that there was an absence of clinical symptoms and only a mild depression of plasma copper, Cp, and hemoglobin.

For the most part, studies have not included the more clinically prevalent marginal dietary deficiencies. Therefore, this reseach project was designed to examine two levels of marginal dietary magnesium and copper deficiencies, in addition to severe deficiencies, on immune function. Furthermore, the interaction of magnesium and copper on immunocompetence is unknown. The present research attempts to describe those effects.

In the next chapter, the procedures and analytic methods followed for each experiment in this research will be explained.

Chapter 3

METHODOLOGY

In order to determine the effects of dietary magnesium deficiency, dietary copper deficiency, and a combined magnesium and copper deficiency on immune function, this study was divided into three experiments. First, the general protocol and analytic methods followed for all experiments will be discussed. Then, the procedures unique to each experiment will be detailed.

General Animal Management Procedures

Male Sprague Dawley rats with an initial weight of approximately 100 gm were used for each experiment. The rats were individually housed in stainless-steel, wire-bottom cages. A 12/12 hour light/dark cycle was maintained throughout the experiments. The rats received a commercial diet for one week after shipping to adjust to the new environment. Rats then were randomly assigned to treatment diets as described for each experiment.

Purified diets were prepared to be adequate in all nutrients, except for magnesium and copper, based on recommendations of the American Institute of Nutrition (1977). Composition of the basal diet is shown in Table 1. Appropriate amounts of magnesium and copper were added for each experiment based on requirements set by the National

Table 1. Basal diet formulation based on the AIN-76[a] diet recommendations.

Composition	percent
Casein, purified high nitrogen	20.0
DL-Methionine	0.3
Corn starch	32.5
Sucrose	32.5
Non-nutritive fiber	5.0
Corn oil	5.0
AIN custom mineral mix[b]	3.5
AIN 76A vitamin mix[c]	1.0
Choline bitartrate	0.2
BHT	(0.015% of oil)

[a] American Institute of Nutrition (1977)

[b] composition: calcium phosphate (500.00 gm/kg of mixture), sodium chloride (74.00 gm/kg), potassium citrate (220.00 gm/kg), potassium sulfate (52.00 gm/kg), magnesium oxide (24.00 gm/kg for adequate diets), manganous carbonate (3.50 gm/kg), ferric citrate (6.00 gm/kg), zinc carbonate (1.60 gm/kg), cupric carbonate (0.30 gm/kg for adequate diets), potassium iodate (0.01 gm/kg), sodium selenite (0.01 gm/kg), chromium potassium sulfate (0.55 gm/kg), sucrose (118.00 gm/kg).

[c] composition: thiamine HCl (600.0 mg/kg mixture), riboflavin (600.0 mg/kg), pyridoxine HCl (700.0 mg/kg), nicotinic acid (3.0 gm/kg), D-calcium pantothenate (1.6 gm/kg), folic acid (200.0 mg/kg), D-biotin (20.0 mg/kg), cyanocobalamin (1.0 mg/kg), retinyl palmitate, 250,000 IU/gm (1.6 gm/kg), DL-alpha-tocopheryl acetate, 250 IU/gm (20.0 gm/kg), cholecalciferol, 400,000 IU/gm (2.5 mg/kg), menaquinone (500 ug/kg), sucrose (972.9 gm/kg).

Research Council (1978). The diets were prepared in 5 kg batches at the start of each experiment and stored in sealed, black plastic bags.

The powdered diets were placed in special feeders to minimize spillage during consumption. The feeders consisted of small glass jars secured to saucers to prevent tipping and to catch spilled feed. Openings in the jar lids were cut so that the rats could reach into the jar to eat, but not be able to scoop out the feed.

The feed and deionized water were provided ad libitum except to rats in the pair-fed groups. The rats were fed every other day and the amount fed was recorded. After two days, the remaining feed was weighed and the amount of food consumed was calculated. Rats in the pair-fed groups were fed daily, and consumption was recorded daily.

On the first day of each experiment, two rats per treatment group were weighed, a blood sample was obtained, and the rats were placed on the test diet. On each of the following three or four days, two rats per treatment were started on the experiment. All succeeding sampling was maintained on the same daily schedule. This schedule was maintained because a maximum of six samples per day could be evaluated with the cellular immunology and phagocytic function assays. Experimental error due to sampling days was then considered in the statistical analysis.

At two-week intervals, the rats were weighed and blood samples were obtained to monitor the nutritional status. The

rats were anesthetized with an intramuscular injection of a 1:10 dilution of Innovar-Vet^{R} (Pitman-Moore, Inc., Washington Crossing, NJ), at a dosage of 0.1 ml/100 gm body weight. Blood samples were obtained by cardiac puncture, and were collected in syringes with preservative-free sodium heparin, 10 U/ml blood (Sigma Chemical Co., St. Louis, MO).

At the midpoint of each experiment, half of the rats in each treatment group were euthanized, while the remaining rats were euthanized at eight weeks. Blood samples were obtained at the time of euthanasia for nutritional and immunological analyses. The spleen and thymus from all rats were removed and weighed.

The nutritional status of the rats was monitored by weight gain and feed efficiency, hematocrit and hemoglobin concentration, plasma magnesium or copper concentration, and bone magnesium or liver copper concentration.

The immune profile included cellular, humoral, and phagocytic analyses. Cellular immunity was assessed by total and differential counts of white blood cells, T- and B-cell quantitation, and lymphocyte stimulation tests. Humoral immunity was assessed by antibody response to heterologous red blood cells, IgM and IgG quantitation, and serum electrophoresis. Bacterial phagocytosis and killing by neutrophils also were included in the immunological analyses.

In addition to determination of the nutritional status and immune function, blood samples from rats consuming the

copper deficient or combined magnesium and copper deficient test diets were analyzed for the enzymes ceruloplasmin, erythrocyte catalase, superoxide dismutase, and myeloperoxidase.

General Analytical Methods

Nutritional Assessment

Whole blood samples collected every two weeks were used to determine hematocrit and hemoglobin (Hemoglobinometer, Coulter Electronics, Inc., Hialeah, FL). The samples then were centrifuged to collect the plasma which was frozen at 0°C until mineral analyses were conducted. Plasma magnesium and copper determinations were made by atomic absorption spectroscopy with an air-acetylene flame (Perkin-Elmer, Norwalk, CN).

Plasma was diluted 1:40 with distilled water for magnesium determinations. Standard magnesium solutions were prepared in distilled water to contain 1, 2 and 3 ug magnesium/ml, then were diluted 1:40 to obtain a standard curve. Standards and samples were aspirated and read in triplicate at a wavelength of 285.2 nm.

Plasma for the copper determinations was diluted 1:1 with distilled water. Standard copper solutions were prepared in distilled water to contain 0.5, 1.0 and 2.0 ug copper/ml, then were diluted 1:1 with 10% glycerol to match the viscosity of the plasma. Due to the small amount of plasma available for copper analysis, a Teflon sampling cup

was used for aspirating 100 ul of the standards and samples (Manning, 1975). Readings were made in triplicate at a wavelength of 324.8 nm.

Immunological Assessments

Tests for immune function were conducted on samples collected at the midpoint and the final weeks of each experiment. The rats were anesthetized with Innovar-Vet®, and 5 to 7 ml blood was collected by cardiac puncture in preservative-free heparinized syringes. The rats then were euthanized with pentobarbitol. The spleen and thymus were removed and weighed.

For each rat, the blood sample was divided as follows: two 2 ml samples were placed into sterile capped polystyrene tubes for immunological assays, and 1 to 2 ml samples were used for nutritional analyses as previously described. Total white blood cell counts were obtained with a semi-automated cell counter (Coulter Counter, Coulter Electronics, Inc., Hialeah, FL). Blood smears for the differential counts were prepared using a modification of the Wright's stain technique (LeukoStat, Fisher Diagnostics, Orangeburg, NY).

Cell Separation Technique

The immunologic samples were certrifuged at 680 x g for 10 minutes, and the plasma was removed and refrigerated for use in the humoral assays. The cells were resuspended with 0.9% saline to 20 ml volumes, layered on 3 ml ficoll, sodium

diatrizoate, 1.077 gm/ml (Ficoll Histopaque^{R}-1077, Sigma Chemical Co., St. Louis, MO) in conical polystyrene tubes, and centrifuged at 410 x g for 30 minutes at room temperature. The saline-ficoll interface layer containing the lymphocytes was collected for the cellular assays.

The red blood cell pellet, containing the neutrophils, was resuspended with 8 ml 0.9% saline and layered on 4 ml colloidal PVP coated silica, 1.087 gm/ml (Percoll^{R}, Sigma Chemical Co., St. Louis, MO) in polystyrene tubes. After centrifugation at 410 x g for 15 minutes at room temperature, the neutrophils were withdrawn at the saline-Percoll interface for the phagocytic assay.

Cellular Assays

The methods for assessing cellular immunity were conducted at the midpoint and final weeks of each experiment. Total and differential white blood cell counts also were determined from samples collected at the initiation of the experiment to provide baseline data. The lymphocytes collected after Ficoll gradient separation (See: Cell Separation Technique) were used for T- and B- cell quantitation and mitogen stimulation.

T- and B-cell Quantitation

Surface marker assays were used to quantitate T- and B-cells (Lo and McClure, 1984). T cells were identified by Arachis hypogaea peanut agglutinin (PNA) conjugated to

fluorescein (FITC), which bound to D-galactose on the cell surfaces. B cells were identified by an anti-rat Ig-FITC conjugate.

The isolated lymphocytes (See: Cell Separation Technique) were washed in 20 ml calcium and magnesium free (CMF) Hanks balanced salt solution, pH 7.4, (GIBCO Laboratories, Grand Island, NY), and centrifuged at 280 x g for 10 minutes. This procedure was repeated three times. The supernatant was removed, and 1.5 ml of RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY) and 0.5 ml fetal calf serum (FCS) were added to the cell pellet. To label phagocytic cells, 100 ul of a 1% suspension of latex beads (75 um in diameter) was added and thoroughly mixed. The cell-latex suspension then was incubated in a 37°C water bath for 30 minutes, and mixed at 10 minute intervals.

After incubation, the cell-latex suspension was layered over 1 ml FCS, centrifuged at 210 x g for five minutes, and the supernatant was removed. The cell pellet was resuspended in 2 ml phosphate buffered saline (PBS), pH 7.4, with 5% FCS, layered over 1 ml FCS, and centrifuged at 210 x g for five minutes. The supernatant was removed, 1 ml RPMI was added to the cell pellet, and the cells were counted and adjusted to a concentration of 1 x 10^7 cells/ml with RPMI. An aliquot of cells was stained with PNA-FITC (EY Laboratories, San Mateo, CA), while a separate aliquot of cells was stained with goat anti-rat IgG-FITC (CopperBiomedical, Inc., West Chester, PA).

A 1:2 dilution of the PNA-FITC conjugate was prepared with PBS containing 0.02% sodium azide, and 0.1 ml was added to 0.3 ml of the cell suspension (3 x 10^6 cells). The mixture was incubated at 4°C for 30 minutes. One ml PBS solution containing 0.02% sodium azide and 5% FCS was added, mixed, and then centrifuged at 500 x g for five minutes. The supernatant was removed, and the previous wash was repeated.

Next, the cell pellet was resuspended in 100 ul of the PBS, 0.02% sodium azide, 5% FCS solution, and was kept at 4°C in the dark until viewed and counted under a fluorescent microscope. A total of 200 latex-negative mononuclear cells were counted. Each field was examined using alternating bright-field and fluorescent microscopy with a Leitz Dialux phase contrast and epiiluminescence microscope, H_2 filter package (Dolen, Scientific, Houston, TX). The fluorescent-positive cells were considered T cells, and were expressed as percent of total cells counted.

To the other 0.3 ml aliquot of cells (3 x 10^6 cells), 0.1 ml goat anti-rat IgG-F(ab')^2 FITC conjugate was added. The conjugate was diluted 1:8 with PBS containing 0.02% sodium azide. The dilution was determined previously, and was two dilutions below that at which the number of fluorescent cells began to decrease. The cell mixture was incubated, washed, and counted as described above for the PNA procedure. Latex-negative, fluorescent-positive cells were considered B cells.

Lymphocyte Stimulation

Lymphocyte proliferation was analyzed using mitogen stimulation, and was quantitated by measuring thymidine incorporation into DNA (Barta, et al., 1984a). Peripheral blood lymphocytes were treated with the following mitogens: phytohemagglutinin (PHA), an effector T cell mitogen; Concanavalin A (Con A), a suppressor T cell mitogen; and pokeweed mitogen (PWM), a general T- and B cell mitogen.

The isolated lymphocytes (See: Cell Separation Technique) were washed in 20 ml calcium and magnesium free (CMF) Hanks balanced salt solution, pH 7.4, (GIBCO Laboratories, Grand Island, NY), and centrifuged at 280 x g for 10 minutes. This procedure was repeated three times. The supernatant was removed, 1 ml RPMI-1640 medium (GIBCO Laboratorie, Grand Island, NY) was added to the cell pellet, and the cells were counted and adjusted to a concentration of 1 x 10^7 cells/ml with RPMI.

Lymphocyte cultures were prepared in sterile multi-well flat-bottom microtiter plates. Each well received 100 ul of 20% fetal calf serum in RPMI medium, and 100 ul of the lymphocyte suspension. Three ug of PHA, Con A, or PWM were added to selected wells, while control cultures received no mitogen. Cultures were prepared in triplicate. The plates were covered and incubated for 72 hours at 37°C in a humidified atmosphere containing 5% CO_2.

One uCi [^{3}H]thymidine (6.7 mCi/mmole) in RPMI was added to each well for the last 16 hours of the 72-hour incubation

period. The samples were harvested onto glass filters using a multisample harvester. The filters were dried, then placed into scintillation vials. Two ml of scintillation cocktail were added to each vial. The samples were counted in a beta-scintillation counter (Tricarb 4640, Packard Instrument Co., Downers Grove, IL). The results were reported as counts per minute (CPM) and as a stimulation index (SI). The SI was calculated as the CPM of cultures with mitogen/CPM of cultures without mitogen.

Humoral Assays

Plasma samples obtained before the rats were placed on the test diets were used to assess humoral immunity. Then, the same procedures were used on the plasma samples from the rats euthanized at either the midpoint or at the end of the experiment. All plasma samples were refrigerated until assayed. Initial samples provided a baseline measure of immunoglobulin status before antigen challenge. The rats were immunized with a photometrically standardized 0.5% suspension of washed sheep red blood cells (sRBC) six days prior to euthanasia (Garvey, et al., 1977). A single intraperitoneal injection was given at 0.1 ml/25 gm body weight.

Antibody Response

Antibody response was measured by a hemagglutination technique (Garvey, et al., 1977), using a 1% suspension of

sRBC. All plasma samples were heated for 30 minutes in a 56°C water bath to inactivate complement. To each well in a multi-well round-bottom plate, 50 ul phosphate diluent, 0.11 M NaH_2PO_4-Na_2HPO_4 with 0.2% w/v gelatin, pH 7.4, was added.

Next, to the first well, 50 ul of either undiluted plasma of prechallenge samples or 1:5 diluted plasma of postchallenge samples was added. The samples were serially diluted 2-fold to a 1:256 dilution for prechallenge samples and 1:640 for postchallenge samples. Fifty ul phosphate diluent plus 25 ul sRBC suspension were added to each well. The plates were sealed and then incubated at 37°C overnight.

The samples were observed for agglutination, and titers were recorded. Titer was determined as the reciprocal of the greatest dilution of plasma where agglutination was observed. When the titers of postchallenge samples were less than 10, the assay was repeated using undiluted samples.

IgG and IgM Quantitation

IgG and IgM were quantitated by rocket immunoelectrophoresis (Barta, et al., 1984), a technique based on antigen-antibody precipitation. During electrophoresis, the rat immunoglobulins migrated through a gel containing goat anti-rat IgG or IgM antisera. Precipitation occurred at the point of optimal antigen-antibody proportions. The area of the rocket-shaped

precipitate was quantitated by comparison with standard solutions of rat immunoglobulin.

A 1% agarose gel was prepared by dissolving 1 gm agarose, Type 1 low EEO, (Sigma Chemical Co., St. Louis, MO) in 100 ml 0.015 M barbital buffer, pH 8.6, conductivity 0.86 mS, and was refrigerated until needed. GelBond™ film (FMC BioProducts, Rockland, ME) was cut into a plate size of 8 x 20.5 cm and placed elevated on a leveling table.

Volumes of agarose gel, 1.3 ml/cm width for IgG assays and 1.8 ml/cm width for IgM, were warmed as needed in a 56°C water bath. Undiluted antiserum, 160 ul goat anti-rat IgG or 360 ul goat anti-rat IgM (Cappel Laboratories, CopperBiomedical, Inc., Malverin, PA), was gently mixed into the gel and carefully poured on the GelBond™ film. The film was allowed to set 40 minutes, and was kept in a humid chamber until used.

Wells were punched into the gel film allowing for an 8 mm distance between the centers. Well sizes used were 2 mm for IgG determination and 3 mm for IgM. The BioRad (model 1400) gel electrophoresis horizontal buffer chambers were filled with 0.025 M barbital buffer, pH 8.6, conductivity 1.32 mS, and the cooling system was set to maintain 17°C. The gel plate was placed on the cooling plate with the wells nearer the cathode. Four layers of #2 Whatman filter paper were placed on each side of the gel film and into the buffer.

Rat IgG standards (Pel-Freez Biologicals, Rogers, AR) or IgM standards (Calbiochem Biochemicals, San Diego, CA) and the samples were added to the wells. The voltage was applied and maintained at 7 volts per centimeter (28 volts total) for three hours. After electrophoresis, the film was removed, covered with filter papers, and pressed overnight.

The film then was washed in 0.3 M NaCl for 30 minutes, followed by 0.15 M NaCl for 30 minutes. The film was pressed under filter papers for 15 minutes, then rinsed with distilled water, and allowed to dry in a 60°C oven. The precipitated proteins were fixed for 10 minutes in a freshly prepared destain solution consisting of 90 ml methanol, 90 ml distilled water, and 20 ml acetic acid. The proteins then were stained using Coomassie blue (Sigma Chemical Co., St. Louis, MO) for 20 minutes, followed by successive destaining until the film background was clear.

The stained "rockets" were enlarged on a projection screen and traced onto paper. The areas were measured using a planimeter, and the concentrations of immunoglobulin in the samples were determined from the standard curve obtained from the known concentrations of IgG or IgM.

Electrophoresis

Plasma alpha, beta, and gamma globulins were quantitated by cellulose acetate electrophoresis (Barta and Pourciau, 1984). A Tris-barbital, sodium barbital buffer (Electra® HR buffer, Helena Laboratories, Beaumont, TX),

pH 8.8, ionic strength 0.05, was prepared. Cellulose acetate plates were soaked in buffer for 20 minutes. The electrophoresis chamber was prepared by pouring 50 ml buffer into each outer compartment. Paper wicks were wetted in the buffer, then draped over the support bridges.

Sample well plates were filled with 6 ul of sample, and then covered with a glass slide. A saturated cellulose acetate plate was removed from the buffer, blotted, and placed on an aligning base. The samples were applied to the cellulose acetate plate using a sample applicator. The plate then was placed on the wicks in the chamber, and electrophoresed at 180 volts for 15 minutes.

The cellulose acetate strips were stained in 0.5% Ponceau-S (Helena Laboratories, Beaumont, TX) in 7.5% trichloroacetic acid for six minutes and destained three times in 5% acetic acid washes. The strips were blotted and dehydrated in methanol for 10 minutes.

Next, the strips were soaked for five minutes in a freshly prepared clearing solution consisting of 15 ml glacial acetic acid, 35 ml anhydrous methanol, and 2 ml polyethylene glycol. The strips were dried in a 60°C oven until clear. The strips were scanned in a densitometer (Quick Scan, Helena Laboratories, Beaumont, TX) at 525 nm to determine the percentage of individual proteins. The concentrations of the plasma proteins were calculated from total protein determined by the Lowry procedure (Lowry, et al., 1951).

Phagocytic Assay

Bacterial phagocytosis and killing were evaluated on neutrophils with a method that utilized an acridine orange (AO) stain (Smith and Rommel, 1977; Enright and Jeffers, 1984). The technique allowed phagocytic and bactericidal activity to occur in a suspension of neutrophils and bacteria. After staining with AO, the neutrophils were observed under a fluorescent microscope. AO would combine with DNA and fluorese green, and with RNA or denatured DNA and fluorese red. Thus, viable phagocytized bacteria emitted green, while killed bacteria appeared red.

The neutrophils collected after Percoll^{R} separation (See: Cell Separation Technique) were washed twice with 0.9% saline in polypropylene conical tubes, and then resuspended in 1 ml Dulbeccos phosphate buffered saline (DPBS), pH 7.4 (GIBCO Laboratories, Grand Island, NY). Neutrophils were counted in a hemocytometer and adjusted to a concentration of 1 x 10^{6} cells/ml with DPBS. Cell viability was checked using trypan blue exclusion, and usually was greater than 95%.

Fresh E. coli cultures incubated at 37°C overnight on nutrient agar plates were harvested with DPBS, and washed two times. The cells were resuspended in DPBS to a concentration of 1 x 10^{8} cells/ml, determined spectrophotometrically at 650 nm with an absorbance of 0.250, then were diluted for a final concentration of 1 x 10^{7} cells/ml.

Equal volumes (0.5 ml) of the neutrophil and bacteria suspensions were combined in polypropylene test tubes, and incubated in a 37°C shaker water bath for 90 minutes. After incubation, 400 ul of cool 0.014% acridine orange was added to each sample, gently mixed, and allowed to stand at room temperature for one minute. The samples were centrifuged at 200 x g for five minutes. The supernatant was discarded, and the pellets were resuspended in 100 ul DPBS.

Phagocytosis and killing were examined on a Zeiss epi-fluorescent, darkfield microscope, 100-watt Halogen illumination, FITC filter package (#48 77 09). A total of 200 neutrophils were counted, and were classified as: 1) without ingested E. coli; 2) phagocytized E. coli, but did not kill; or 3) phagocytized and killed E. coli. Results were expressed as percentages for each classification.

Procedures Unique to Each Experiment

Experiment 1: Magnesium Deficiencies

Forty male Sprague Dawley rats, 145 gm initial weight, were used in the experiment designed to determine the effects of dietary magnesium deficiency on immune function. Four levels of magnesium were used to achieve: 1) an adequate level of 400 ug/gm of the diet; 2) a mild deficiency of 280 ug/gm; 3) a moderate deficiency of 160 ug/gm; and 4) a severe deficiency of 50 ug/gm. Magnesium oxide was added to the mineral mixture, as listed in Table 2 to achieve these levels. Actual magnesium concentrations

Table 2. Magnesium concentration of each diet treatment in Experiment 1.

Target diet (ug Mg/gm)	MgO added[a] (mg/kg of total feed)	Actual diet (ug Mg/gm)
400	618.4	392.7
280	419.2	249.8
160	220.0	140.8
50	37.4	58.9

[a] The basal diet without magnesium in the mineral mixture contained 27.5 ug magnesium/gm diet. MgO was added to the mineral mixture, which then was mixed with the other feed ingredients.

of the total feed mixtures were determined by atomic absorption spectroscopy following nitric acid digestion.

Eight rats were randomly assigned to each treatment group. A fifth treatment group consisted of eight rats that consumed the 400 ug magnesium/gm diet, but were pair fed to the rats consuming the 50 ug magnesium/gm diet. The amount of feed consumed by each rat on the 50 ug magnesium/gm diet was recorded on a daily basis and that amount was fed the following day to its paired mate in the 400 ug magnesium/gm pair-fed treatment group.

All rats were weighed, and blood samples were obtained before being placed on the treatments, and at week two. Four rats from each treatment were euthanized at week three. The remaining rats were weighed and bled at week four and week six, and then were euthanized at week eight. At the time of euthanasia, samples were obtained from all rats as described in the General Animal Management Procedures section. Moreover, the femur was removed and frozen until analyzed for bone magnesium content. Nutritional and immunological assessments were conducted, as described in the General Analytical Methods section.

Bone Magnesium Assay

Bone magnesium in femur diaphyseal sections was analyzed as described by Hunt (1969). The bones were cleaned of all adhering tissue and split longitudinally. The bone marrow was removed, and each fragment was cleaned with

0.9% saline. The bone fragments were immersed in ether for 10 minutes, then allowed to stand at room temperature for 10 minutes. The wet weight of the bone was recorded. The bone fragments were dried at 90°C to a constant weight. After cooling in a dessicator, the dry weight was recorded.

The bones were ashed in a muffle furnace for 16 hours at 600°C. After removal from the furnace, 2 ml 5 N HCl were added to each sample and heated until the bone dissolved. Bone samples were diluted 1:2500 with 0.5% lanthanum chloride in distilled water. Magnesium concentrations were determined by atomic absorption spectroscopy.

Experiment 2: Copper Deficiencies

Fifty male Sprague Dawley rats, 100 gm initial weight, were used in the experiment designed to determine the effects of dietary copper deficiency on immune function. Four levels of copper were used to achieve: 1) an adequate level of 5.0 ug/gm of the diet; 2) a mild deficiency of 3.5 ug/gm; 3) a moderate deficiency of 2.0 ug/gm; 4) and a severe deficiency of 0.5 ug/gm.

Mineral mixtures were prepared (ICN Nutritional Biochemicals, Cleveland, OH) to contain copper at the following levels: 126 ug/gm, 83 ug/gm, 40 ug/gm, and 0 ug/gm; and were added to the feed mixtures, as listed in Table 3. Actual copper levels of the feed mixtures were determined by atomic absorption spectroscopy following nitric acid digestion.

Table 3. Copper concentration of each diet treatment in Experiment 2.

Target diet (ug Cu/gm)	Cupric carbonate[a] (mg/gm of mineral mix)	Actual diet (ug Cu/gm)
5.0	126	5.20
3.5	83	3.72
2.0	40	2.10
0.5	0	0.59

[a] The basal diet without copper in the mineral mixture contained 0.5 ug copper/gm diet. The custom mineral mixtures were 3.5% of the diet.

Ten rats were randomly assigned to each treatment group. A fifth treatment group consisted of 10 rats that consumed the 5.0 ug copper/gm diet, but were pair fed to the rats consuming the 0.5 ug copper/gm diet. The amount of feed consumed by each rat on the 0.5 ug copper/gm diet was recorded on a daily basis and that amount was fed the following day to its paired mate in the 5.0 ug copper/gm pair-fed treatment group.

Several rats in the treatment group with 0.5 ug copper/gm died during the experiment, so their paired mates in the 5.0 ug copper/gm treatment group were then fed the average amount of feed consumed by the remaining rats in the 0.5 ug copper/gm treatment group.

All rats were weighed and blood samples were obtained before being placed on the diets and at week two. Five rats from each treatment were weighed and euthanized at week four. The remaining rats were weighed and bled at week four, and at week six. Rats on the 0.5 ug copper/gm diet were not bled at this time because unexpected deaths occurred at week four from the cardiac puncture.

The rats were euthanized at week eight. At the time of euthanasia, samples were obtained from all rats, as described in the General Animal Management Procedures section. Moreover, the liver was removed, weighed, and frozen until analyzed for copper content. Nutritional and immunological assessments were conducted, as described in the General Analytical Methods section.

Ceruloplasmin (Cp) was assessed as an additional parameter of copper status for all rats. The effect of Cp's ferroxidase activity on iron status then was monitored by erythrocyte catalase activity at four and eight weeks. Lastly, the effect of copper status on neutrophil superoxide dismutase (SOD) and myeloperoxidase (MPO) were studied. These two enzymes are involved in the bactericidal activity of neutrophils. Measurements were performed on the enzymes from neutrophils separated during the phagocytic assay.

Liver Copper Assay

A wet nitric acid technique was used to digest liver samples (Carthew and Dey, 1985). Approximately 1 gm liver samples were weighed and placed into Teflon digestion vials with 3 ml of 8 M nitric acid. The capped vials were incubated in a 90°C water bath for 45 minutes, cooled, and transferred to test tubes. Loss of volume due to evaporation was negligible. After cooling, fat particles that rose to the top were removed by pipet. The clear solution then was diluted 1:2 with distilled water for a final dilution of 1:6, and copper concentrations were determined by atomic absorption spectroscopy.

Ceruloplasmin

Ceruloplasmin (Cp) activity was determined spectrophotometrically, as described by Lehman et al. (1974), using o-dianisidine as the substrate. Plasma samples

were frozen at 0°C until assayed. Two test tubes per sample, containing 0.7 ml of 0.1 M acetate buffer, pH 5.0, and 0.05 ml of plasma, were incubated in a shaking water bath at 30°C. To each tube, 0.2 ml o-dianisidine reagent (250 mg/100 ml) was added.

For one set of samples, the reaction was stopped after five minutes by the addition of 2 ml of 9 M sulfuric acid, while the reaction in the second set of samples was stopped after 15 minutes. The absorbances were measured at 540 nm and enzyme activity was calculated from the formula:

$$(A_{15} - A_5) \times 6.25 \times 10^2 \text{ mU/ml},$$

where A_{15} is the absorbance after 15 minutes and A_5 is the absorbance after five minutes.

Erythrocyte Catalase

Whole blood samples obtained from the rats euthanized at the midpoint and at the end of the experiment were measured for catalase activity. The blood samples were refrigerated at 4°C until assayed within seven days of collection. The procedure (Aebi, 1984; Aebi and Suter, 1969) followed the decomposition of hydrogen peroxide by catalase spectrophotometrically in the ultraviolet range.

Two ml whole blood samples were centrifuged, then the plasma and white cell layer were removed. The red cells were washed twice in 0.9% saline, then a stock hemolysate of each sample was prepared using 0.5 ml packed cells and 1 ml distilled water. The hemoglobin content of the stock

hemolysate was determined in duplicate (Total Hemoglobin Procedure No. 525, Sigma Diagnostics, St. Louis, MO) based on the method by Drabkin and Austin (1935).

Immediately before the catalase assay was performed on each sample, a 1:1000 dilution was made of the stock hemolysate with 50 mM phosphate buffer, pH 7.0. The sample cuvette containing 1 ml of diluted hemolysate, and 1 ml phosphate buffer was read at a wavelength of 240 nm against a control cuvette containing 1 ml of diluted hemolysate, 1 ml phosphate buffer and 1 ml distilled water. The reaction was started in the sample cuvette with the addition of 1 ml 30 mM hydrogen peroxide. The decrease in absorbance from an approximate initial absorbance of 0.500 was recorded for a 15-second time interval.

The rate constant (k) related to the hemoglobin (Hb) content served as the measurement of erythrocyte catalase activity. Therefore, the activity was calculated with the formula:

$$k/gm\ Hb = (2.3/15\ sec)(a/b)(\log A_1/A_2),$$

where a is the dilution factor, b is the Hb content of the hemolysate in gm/l, A_1 is the absorbance at time = 0, and A_2 is the absorbance at time = 15 seconds.

Superoxide Dismutase

For the SOD assay, isolated neutrophils (See: Cell Separation Technique, General Analytical Methods section) were suspended in DPBS at a concentration of 1×10^6

cells/ml, were frozen and thawed three times, then were sonicated 10 seconds in an ice bath to disrupt the cell membranes. The samples were centrifuged at 13,000 x g in a microcentrifuge for 15 minutes. The supernatant was assayed which contained the cuprozinc SOD from the cytosol fraction (Salin and McCord, 1974).

The ferricytochrome c reduction assay developed by McCord and Fridovich (1969) was used in which a xanthine-xanthine oxidase system was utilized as a source for the free radical $O_2 \cdot ^-$. The reduction rate of cytochrome c was spectrophotometrically monitored as SOD competed for the superoxide radicals.

The procedure (Flohe and Otting, 1984) with modifications to increase sensitivity (Salin and McCord, 1974) defined one unit of SOD as that amount of enzyme required to inhibit the rate of cytochrome c reduction by 50 percent. A standard calibration curve was obtained using purified bovine superoxide dismutase (Sigma Chemical Co., St. Louis, MO) with SOD standard concentrations plotted against the reciprocal absorbance change per minute of cytochrome c reduction.

Two solutions were prepared for the assay as follows: Solution A was made by mixing 0.76 mg of 5 uM xanthine and 6.2 mg of 0.5 uM cytochrome c into 10 ml of 0.001 N NaOH, which then was added to 100 ml 20 mM sodium carbonate, pH 10.0, containing 0.1 mM EDTA. Solution B was a freshly prepared solution of xanthine oxidase in 0.1 mM EDTA, with

approximately 0.2 U/ml. The SOD standard or sample supernatant (100 ul) was added to 2.8 ml Solution A. The addition of 100 ul Solution B started the reaction and the change in absorbance per minute was recorded at 418 nm.

To obtain a measurement of the cuprozinc SOD in the samples, which also contained some mangano-SOD, 1 mM sodium cyanide (10 ul) was added to the reaction mixture (Beauchamp and Fridovich, 1973). The cuprozinc enzyme was inhibited by the cyanide. Therefore, the cuprozinc SOD was calculated from the total SOD measured without cyanide minus the manganoenzyme measured with cyanide.

Myeloperoxidase

Myeloperoxidase (MPO) also was measured in the neutrophils collected from the rats euthanized at the midpoint and at the end of the experiment. The isolated cells were suspended at a concentration of 1 x 10^6 cells/ml in a solution containing 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM potassium phosphate buffer, pH 6.0. The cells were sonicated for 10 seconds in an ice bath, freeze-thawed three times, then sonicated again. The samples were centrifuged at 13,000 x g in a microcentrifuge for 15 minutes, and the supernatant was used for the assay.

MPO activity was determined spectrophotometrically using o-dianisidine as the substrate (Bradley, et al., 1982a; 1982b). The assay solution consisted of 50 mM

phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide.

The supernatant from each sample (0.1 ml) was added to 2.9 ml assay solution, mixed, and the change in absorbance at 460 nm was measured for one minute. The amount which degraded one micromole peroxide per minute was defined as one unit MPO activity. Therefore, the activity was calculated as follows:

$$MPO \times 10^{-7}\ U/neutrophil = A/3.76 \times 10,$$

where A is the change in absorbance per minute.

Experiment 3: Magnesium and Copper Deficiencies

Sixty male Sprague Dawley rats, 100 gm initial weight, were used in the experiment designed to determine the effects of a combined dietary magnesium and copper deficiency on immune function. One treatment consisted of severely deficient levels of magnesium (50 ug/gm) and copper (0.5 ug/gm). The two marginal levels of magnesium, 160 ug/gm and 280 ug/gm, and the two marginal levels of copper, 2.0 ug/gm and 3.5 ug/gm, were combined in a 2 x 2 factorial arrangement. The control treatment consisted of adequate magnesium (400 ug/gm) and copper (5.0 ug/gm). Ten rats were randomly assigned to each treatment group.

Mineral mixtures without magnesium were prepared to contain copper at four levels: 126 ug/gm, 83 ug/gm, 40 ug/gm, and 0 ug/gm (ICN Nutritional Biochemicals, Cleveland, OH). Magnesium oxide was added to the mineral

mixtures in the appropriate amounts. The mineral mixtures then were added to the feed mixtures, as listed in Table 4. Actual magnesium and copper levels of the total feed mixtures, determined by atomic absorption spectroscopy following nitric acid digestion also are listed in Table 4.

All rats were weighed and blood samples were obtained before being placed on the diets and at week two. Five rats from each treatment were euthanized at week four. The remaining rats were weighed at weeks four and six, but due to unexpected mortality in the previous experiment with copper deficiencies, blood samples were not obtained. The rats were euthanized at week eight.

At the time of euthanasia, samples were obtained from all rats, as described in the General Animal Management Procedures section. Moreover, the femur and liver were collected and analyzed, as indicated in Experiments 1 and 2, respectively. Nutritional and immunological assessments were conducted (See: General Analytical Methods section). Catalase, Cp, SOD and MPO also were assessed which were outlined in the section for Experiment 2.

Statistical Analyses

The nutritional data were analyzed as a split plot design. Dietary treatments were considered the whole plots while sampling time-periods were considered the subplots (Steel and Torrie, 1980). The whole plot error term was the variance among the rats within diets.

Table 4. Magnesium and copper concentration of each diet treatment in Experiment 3.

Target diet (ug/gm)		Mineral added[a]		Actual diet (ug/gm)	
Mg	Cu	Mg[b]	Cu[c]	Mg	Cu
400	5.0	618.4	126	401.0	6.21
280	3.5	419.2	83	300.8	3.26
160	3.5	220.0	83	168.1	3.25
280	2.0	419.2	40	311.4	1.98
160	2.0	220.0	40	175.5	1.90
50	0.5	37.4	0	68.5	0.33

[a] The basal diet without magnesium and copper in the mineral mixture contained 27.5 ug magnesium/gm diet and 0.5 ug copper/gm diet.

[b] MgO (mg/kg of total feed). MgO was added to the mineral mixture, which then was mixed with the other feed ingredients.

[c] $CuCO_3$ (mg/gm of mineral mix). The custom mineral mixtures were 3.5% of the diet.

Data obtained at the midpoint and at the end of each experiment were analyzed as a completely randomized design with a factorial arrangement of treatments (diets x sampling time-periods). A randomized complete block design with a factorial arrangement of treatments was used for cellular immunologic and neutrophil function data. The blocks consisted of sampling days when data were collected from one rat per treatment per day. The pre-test data from the humoral assessments were included as covariates.

Additionally, mitogen stimulation responses and antibody responses were analyzed with regression analysis. Plasma mineral levels were the independent variable and immune responses were the dependent variable (Steel and Torrie, 1980).

The null hypotheses, that treatment means were equal, were tested at the 0.95 level of significance. When the null hypotheses were rejected, least square mean paired t-tests were used to test differences between the treatment means and the interaction means. When the null hypotheses were not rejected based on the F-tests, no further mean comparisons were made.

The analyses were conducted on the data using the general linear model procedure of the statistical analysis system (SAS, 1985).

In the next chapter the results of the three experiments will be presented, followed by Chapter 5 with a summary, discussion and conclusions based on the findings.

Chapter 4

RESULTS

The rats in each experiment showed the characteristic clinical signs associated with copper and/or magnesium deficiencies. Immunological responses were varied, but some trends emerged according to nutritional status as affected by dietary copper or magnesium level. The results, based on least square means, will be reported for each experiment. Statistical significance was based on the analysis of variance computations, and the minimum significance was set at the 0.05 level.

The tables in this chapter show only the significant mean comparisons between the treatments and the controls. When the diet*week interactions in the analysis of variance were not significant, statistical differences were reported only for the main effect means. Information on mean comparisons among all treatments are located in the Appendix, with the summaries of the statistical models.

Experiment 1: Magnesium Deficiencies

The rats were similar before the dietary treatments in weight (158.9±2.2 gm), hemoglobin (13.5±0.2 gm/100 ml), hematocrit (37.8±0.4 percent), and plasma magnesium (2.19±0.04 mg/100 ml). Significant differences among the

dietary treatments and the sampling time-periods then existed after the initiation of the treatments.

Clinical signs of magnesium deficiency began to appear in the rats consuming the low magnesium (50 ug/gm) diet two weeks into the study and remained. These included ulcerative lesions about the head and neck, a coarse hair coat, and skin necrosis. One rat in the 160 ug magnesium/gm treatment group died after heart puncture at week two of the experiment.

Nutritional Status

During the eight-week time period of the experiment, plasma magnesium levels reflected the dietary levels of magnesium ($P<0.001$). Table 5 shows that rats on the 50 ug magnesium/gm diet had the lowest mean plasma magnesium, followed by rats on the 160 ug magnesium/gm diet, the 280 ug magnesium/gm diet, and the 400 ug magnesium/gm ad lib diet. Rats in the pair-fed treatment group had mean plasma magnesium levels similar to the control rats.

Plasma magnesium levels fell at two weeks for all rats, with the greatest decrease in the 50 ug magnesium/gm diet group. Plasma magnesium remained low throughout the study in that group of rats. Although the plasma magnesium concentrations for the rats in the 280 ug magnesium/gm diet group were lower than the controls, they were not significantly different. By eight weeks, the plasma magnesium concentration for the rats in the diet with

Table 5. Least square mean plasma magnesium concentrations (mg/100 ml) for each magnesium dietary treatment at each sampling period (n=8).

Mg Diet ug/gm	Sampling Week Initial	2	3	4	6	8	Mean SE
50	2.12	0.57[c]	0.57[c]	0.53[c]	0.70[c]	0.65[c]	0.82[c] 0.07
160	2.12	1.51[c]	1.44[b]	1.37[b]	1.35[b]	1.89	1.63[c] 0.09
280	2.17	1.84	1.72	1.98	1.69	2.25	1.94 0.07
400, ad lib, control	2.26	2.02	2.05	2.03	1.97	2.11	2.07 0.07
400, pair fed	2.30	1.92	1.93	1.80	2.04	2.17	2.03 0.07
Mean	2.19	1.57	1.54	1.54	1.55	1.81	
SE	0.04	0.04	0.06	0.06	0.06	0.06	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

160 ug magnesium/gm diet group also was not significantly different from that of the control animals.

The rats on the 50 ug magnesium/gm diet had a significantly lower mean hemoglobin concentration than the other treatment groups (Table 6). Hemoglobin values increased by the end of the eight-week time period for all rats, except those on the severely deficient diet. Similar results were found for the hematocrit, but the rats on the 160 ug magnesium/gm diet were similar to the severely deficient rats (Table 7).

Growth of the rats differed according to the diets. As expected, the rats receiving the adequate level of magnesium ad lib had the highest mean weight (281.3$\pm$5.7 gm), followed by the rats on the marginal levels (273.0$\pm$5.7 gm and 272.0$\pm$6.7 gm), with the severely deficient rats having the smallest mean weight (244.0$\pm$5.7 gm). The mean weight of the pair-fed rats (247.1$\pm$5.7 gm) matched that of their mates, illustrating the effect of restricted calories. The difference in weights occurred after the initiation of the treatments, and were statistically significant ($P<0.001$).

The trend in growth also was apparent when total weight gain was considered (Table 8). Rats consuming the adequate diet ad lib had the highest mean weight gain, followed by those on the 280 ug magnesium/gm diet and the 160 ug magnesium/gm diet, respectively. The smallest mean weight gain was for those on the 50 ug magnesium/gm diet. Pair-fed rats had a mean weight gain similar to their matched mates.

Table 6. Least square mean hemoglobin (gm/100 ml) for each magnesium dietary treatment at each sampling period (n=8).

Mg Diet ug/gm	Sampling Week Initial	2	3	4	6	8	Mean SE
50	13.1	14.7	15.6	13.3[b]	16.4[c]	13.1[c]	14.4[c] 0.2
160	13.5	15.6	15.7	15.7	17.3[c]	14.6	15.4 0.3
280	13.6	15.7	14.9	16.6	17.8[b]	15.2	15.6 0.2
400, ad lib, control	13.5	15.3	14.3	15.5	20.3	15.9	15.8 0.2
400, pair fed	13.6	16.1	14.6	15.7	18.3[b]	15.4	15.6 0.2
Mean	13.5	15.5	15.0	15.3	18.0	14.8	
SE	0.2	0.2	0.3	0.3	0.3	0.3	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

Table 7. Least square mean hematocrit (%) for each magnesium dietary treatment at each sampling period (n=8).

Mg Diet ug/gm	Sampling Week Initial	2	3	4	6	8	Mean SE
50	36.9	41.4	43.1	38.4	43.1[c]	38.4[b]	40.2[c] 0.5
160	37.6	43.3	44.9[a]	40.6	35.9[c]	40.9	40.5[b] 0.6
280	37.9	42.9	42.8	46.0[a]	45.3[c]	43.8	43.1 0.5
400, ad lib, control	38.4	42.3	40.4	42.1	51.3	43.8	43.0 0.5
400, pair fed	38.0	43.5	40.8	43.5	47.0[a]	43.3	42.7 0.5
Mean	37.8	42.7	42.4	42.1	44.5	42.0	
SE	0.4	0.4	0.6	0.7	0.7	0.7	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

Table 8. Least square mean weight gain (gm), and feed efficiency (gm weight gain/gm feed intake) for each magnesium treatment at each sampling period (n=4).

	Sampling Week 3		Sampling Week 8		Mean SE	
Mg Diet ug/gm	weight gain	feed eff.	weight gain	feed eff.	weight gain	feed eff.
50	82.0	0.21	153.8	0.16	117.9[c] 8.7	0.18[b] 0.01
160	90.8	0.20	201.3	0.20	146.0 9.4	0.20[a] 0.01
280	106.8	0.23	200.0	0.18	153.4 8.7	0.21 0.01
400, ad lib, control	129.3	0.30	205.3	0.19	167.3 8.7	0.24 0.01
400, pair fed	98.0	0.25	152.5	0.17	125.3[b] 8.7	0.21 0.01
Mean	101.4	0.24	182.6	0.18		
SE	5.5	0.01	5.7	0.01		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

When total feed intake was compared to total weight gain, differences among the treatment groups were found, as also shown in Table 8. Rats on the 50 ug magnesium/gm diet consumed the least amount of food and had low feed efficiency. Their pair-fed mates ate similar amounts of food, but their feed efficiency was higher. This indicated that the magnesium deficiency reduced weight gain not only by a decreased caloric intake, but also by a less efficient utilization of the feed.

The rats in the other groups consumed similar amounts of feed, with the rats in the adequate group having the highest feed efficiency. For all rats, feed efficiency was higher at three weeks than at eight weeks.

Bone magnesium was significantly altered by dietary magnesium as shown in Table 9. The amount of magnesium in the bone also highly correlated with plasma magnesium at three weeks ($r=0.95$, $P<0.001$), and at eight weeks ($r=0.84$, $P<0.001$). The severely deficient diet depleted bone magnesium stores to 58 percent of the adequate levels at three weeks, and to 40 percent at eight weeks ($P<0.001$).

The marginal diets slightly reduced bone magnesium. The levels at three weeks were similar for the 160 ug magnesium/gm diet and the 280 ug magnesium/gm diet. At eight weeks, the 160 ug magnesium/gm diet resulted in lower bone magnesium than the control diet. Both diets with adequate magnesium maintained bone magnesium stores throughout the study.

Table 9. Least square mean bone magnesium (mg/gm bone, dry weight) for each magnesium dietary treatment at each sampling period (n=4).

Mg Diet ug/gm	Sampling Week 3	Sampling Week 8	Mean SE
50	2.79[c]	1.93[c]	2.36[c] 0.08
160	4.33	3.94[c]	4.13[c] 0.08
280	4.47	4.59	4.53 0.08
400, ad lib, control	4.82	4.78	4.80 0.08
400, pair fed	4.73	4.64	4.69 0.08
Mean	4.23	3.98	
SE	0.05	0.05	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

Lymphoid Organs

For comparative purposes, the spleen and thymus weights were expressed as a percent of total body weight (Table 10). Spleen weight negatively correlated with plasma magnesium at three weeks ($r=-0.61$, $P<0.01$) and at eight weeks ($r=-0.78$, $P<0.001$). The severely magnesium deficient rats had significantly enlarged spleens ($P<0.001$). Spleens were 0.30 ± 0.01 percent of the total body weight compared to controls at 0.21 ± 0.01 percent. Rats in all other treatment groups had spleens that were 0.20 ± 0.01 percent of the total body weight. That percentage was higher at three weeks (0.24 ± 0.01 percent), indicating a greater change in body weight than in spleen weight during the eight-week period.

Thymus weight was not significantly altered by dietary magnesium. Again, as total body weight increased, the percentage decreased from 0.23 ± 0.01 percent at three weeks to 0.16 ± 0.01 percent at eight weeks.

Cellular Immunity

Total white blood cell (WBC) counts were negatively correlated with plasma magnesium at three weeks ($r=-0.69$, $P<0.001$). The differences among the treatment groups remained, but because plasma magnesium levels at eight weeks were similar among the marginal and adequate treatment groups, the correlation was not as strong ($r=-0.30$, $P<0.22$).

Table 11 shows that overall, rats consuming the 50 ug magnesium/gm diet had the highest total WBC count, followed

Table 10. Least square mean spleen and thymus weights (gm) as percent of total body weight (gm) for each magnesium dietary treatment at each sampling period (n=4).

Mg Diet ug/gm	Sampling Week 3		Sampling Week 8		Mean SE	
	spleen	thymus	spleen	thymus	spleen	thymus
50	0.30	0.23	0.30	0.15	0.30[c] 0.01	0.19 0.01
160	0.22	0.21	0.19	0.18	0.20 0.01	0.20 0.01
280	0.23	0.25	0.18	0.16	0.20 0.01	0.20 0.01
400, ad lib, control	0.22	0.23	0.20	0.15	0.21 0.01	0.19 0.01
400, pair fed	0.23	0.21	0.18	0.15	0.20 0.01	0.18 0.01
Mean	0.24	0.23	0.21	0.16		
SE	0.01	0.01	0.01	0.01		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

Table 11. Least square mean total white blood cell counts (x 10^3/ul) and differentials for each magnesium dietary treatment for both sampling periods (n=8).

Mg Diet ug/gm	Total WBC	Percent Baso	Percent Eos	Percent Neut	Percent Lymph	Percent Mono
50	12.5[a]	0.2	5.7[b]	17.8	73.0[a]	2.5
SE	1.4	0.1	0.6	2.7	3.0	0.5
160	7.7	0.0	4.3	15.6	77.6	1.8
SE	1.6	0.2	0.7	2.9	3.3	0.5
280	10.4	0.3	2.4	19.9	73.6[a]	2.6
SE	1.4	0.1	0.6	2.7	3.0	0.5
400, ad lib, control	7.2	0.1	2.6	11.2	82.5	2.0
SE	1.4	0.1	0.6	2.7	2.9	0.5
400, pair fed	6.5	0.0	2.4	10.4	84.7	1.5
SE	1.5	0.1	0.6	2.8	3.2	0.5

a, different from control (P<0.05)
b, different from control (P<0.01)
c, different from control (P<0.001)

differentials (x 10^3/ul) based on percent of total WBC:

Mg Diet ug/gm	Baso	Eos	Neut	Lymph	Mono
50	0.0	0.7	2.2	9.1	0.3
160	0.0	0.3	1.2	6.0	0.1
280	0.0	0.2	2.1	7.7	0.3
400, ad lib, control	0.0	0.2	0.8	5.9	0.1
400 pair fed	0.0	0.2	0.7	5.5	0.1

by those in the 280 ug magnesium/gm treatment group. The rats in the 160 ug magnesium/gm treatment group had total WBC counts similar to those in the adequate ad lib and pair-fed groups. The differences could be attributed to an increase in the number of neutrophils and a significant increase of eosinophils.

Dietary treatment had no effect on the percentage of T cells and B cells. Of the total lymphocytes, 63 to 67$\pm$3 percent were T cells, while 31 to 37$\pm$3 percent were B cells.

Lymphocyte stimulation was not affected by changes in dietary magnesium at three and eight weeks. As shown in Table 12, large variation within each dietary treatment group existed. When regression analyses were conducted using plasma magnesium as the independent variable and lymphocyte stimulation response as the dependent variable, no linear or quadratic relationships existed.

Humoral Immunity

Dietary magnesium deficiencies affected total plasma protein, with changes in the albumin and globulin concentrations (Table 13). Significant decreases in plasma proteins was observed with the severely deficient, 50 ug magnesium/gm diet, but not with the marginally deficient diets. At the same time, the restricted caloric intake in the pair-fed treatment did not affect plasma protein. Plasma protein levels increased for all rats during the eight-week

Table 12. Least square mean lymphocyte stimulation responses for each magnesium dietary treatment for both sampling periods (n=8).

Mg Diet ug/gm	PHA		Con A		PWM	
	CPM*	SI**	CPM*	SI**	CPM*	SI**
50	7,356	12.2	47,797	90.3	13,492	21.5
SE	2,927	6.2	17,006	31.0	4,372	7.7
160	5,064	12.6	45,510	121.0	12,495	36.2
SE	3,186	6.9	18,514	33.7	4,760	8.4
280	6,644	12.4	39,972	94.4	6,286	14.4
SE	2,927	6.2	17,006	31.0	4,372	7.7
400, ad lib, control	4,630	15.5	38,964	96.9	11,179	21.6
SE	2,927	6.2	17,006	31.0	4,372	7.7
400, pair fed	8,201	8.6	29,964	31.9	10,800	14.8
SE	2,927	6.2	17,006	31.0	4,372	7.7

*, Counts per minute
**, Stimulation index

Table 13. Least square mean total plasma protein, albumin and globulins (gm/100 ml) for each magnesium dietary treatment for both sampling periods (n=8).

Mg Diet ug/gm	Total Protein	Albumin	Globulins A1	Globulins A2	Globulins Beta	Globulins Gamma	A/G Ratio
50	7.55[a]	3.50[a]	1.15[b]	0.69	2.05	0.21[a]	0.84
SE	0.37	0.21	0.11	0.06	0.12	0.03	0.06
160	9.61	4.62	1.82	0.65	2.31	0.28	1.01
SE	0.40	0.23	0.11	0.07	0.16	0.03	0.07
280	8.36	4.25	1.39	0.54	1.89	0.32	1.00
SE	0.37	0.20	0.10	0.06	0.13	0.03	0.06
400, ad lib, control	8.75	4.21	1.65	0.63	2.01	0.29	0.86
SE	0.37	0.21	0.11	0.06	0.14	0.03	0.06
400, pair fed	8.64	4.41	1.32	0.55	1.90	0.34	1.14[b]
SE	0.37	0.20	0.13	0.06	0.12	0.03	0.06

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

time period with a consistent overall increase in albumin and the globulins.

The lower plasma protein content for the animals receiving the 50 ug magnesium/gm diet could be attributed to lower amounts of albumin, and the alpha 1 and gamma globulins. However, when expressed as a percentage of total protein, the percent albumin, alpha 1 and gamma globulins were not statistically different from the ad lib controls. Instead, the percent of alpha 2 and beta globulins were higher than the control group.

The severely deficient rats also had significantly lower levels of IgM and IgG as indicated in Table 14. Rats consuming marginal levels of magnesium, and those consuming adequate levels but with restricted intakes, were not statistically different from control animals. Yet, IgM levels in all animals correlated with their plasma magnesium concentrations at three weeks ($r=0.63$, $P<0.01$) and at eight weeks ($r=0.57$, $P<0.01$). The rats consuming diets severely deficient in magnesium had mean IgM levels 60 to 70 percent lower than the other animals. No significant changes occurred between the three- and eight-week sampling periods.

Similarly, the severely deficient animals had IgG levels that were approximately half that of the ad lib and pair-fed control animals. In addition, rats consuming 160 ug magnesium/gm had IgG levels that were 62 percent lower than the control group, while those on the 280 ug magnesium/gm diet were similar to the controls. An overall 78 percent

Table 14. Least square mean IgM and IgG (mg/ml) for each magnesium dietary treatment at each sampling period (n=4).

Mg Diet ug/gm	Sampling Week 3 IgM	3 IgG	8 IgM	8 IgG	Mean SE IgM	Mean SE IgG
50	0.96	6.62	0.78	5.72	0.87[b] 0.13	6.17[b] 1.36
160	1.30	9.40	1.30	5.60	1.30 0.14	7.50[a] 1.43
280	1.28	10.86	1.66	11.60	1.47 0.14	11.23 1.33
400, ad lib, control	1.25	12.88	1.46	11.30	1.35 0.12	12.09 1.33
400, pair fed	1.30	16.56	1.48	9.83	1.39 0.12	13.19 1.33
Mean	1.22	11.26	1.34	8.81		
SE	0.08	0.84	0.08	0.87		

a, different from control (P<0.05)
b, different from control (P<0.01)
c, different from control (P<0.001)

decrease in IgG was observed between the three- and eight-week sampling periods for all rats. IgG levels correlated with plasma magnesium at three weeks ($r=0.58$, $P<0.01$), but not at eight weeks ($r=0.04$, $P<0.87$).

When mean comparisons were conducted on the specific antibody response data, large variation contributed to no statistical differences among the diets, although some general trends appeared (Table 15). Mean specific antibody titer for the 50 ug magnesium/gm diet was consistently lower than that of the control, ad lib group.

Analyses also were conducted when antibody was expressed as the log of the reciprocal titer. Severely deficient rats had a mean log antibody response half that of ad lib controls. Mean log titers at three and eight weeks were similar for the ad lib control animals, but were higher at eight weeks for all other dietary groups.

Antibody titer did not exhibit a linear or quadratic relationship with plasma magnesium. At the same time, titers correlated with IgM levels at three weeks ($r=0.49$, $P<0.05$) and at eight weeks ($r=0.45$, $P<0.05$).

Neutrophil Function

Neutrophil phagocytosis of E. coli and intracellular killing was similar for all rats, regardless of diet, as shown on Table 16. More than 30 percent of the neutrophils from rats in each dietary treatment group ingested E. coli. Of those, approximately 90 percent killed the bacteria.

Table 15. Least square mean specific antibody titer and log titer for each magnesium dietary treatment at each sampling period (n=4).

	Sampling Week 3		Sampling Week 8		Mean SE	
Mg Diet ug/gm	Titer	Log Titer	Titer	Log Titer	Titer	Log Titer
50	21	7	13	10	17	8
					44	3
160	53	12	154	20	104	16
					49	4
280	70	16	193	20	131	18
					44	3
400, ad lib, control	130	16	93	17	112	17
					46	3
400, pair fed	18	11	80	17	49	14
					44	3
Mean	58	13	107	17		
SE	28	2	28	2		

Table 16. Least square mean neutrophil phagocytosis and killing (%) for each magnesium dietary treatment for both sampling periods (n=8).

Mg Diet ug/gm	% neutrophils without bacteria	with bacteria, not killed	with bacteria, killed
50	64.3	2.6	33.1
SE	3.1	0.5	3.0
160	66.1	2.9	30.9
SE	3.3	0.6	3.3
280	69.0	2.0	29.0
SE	3.1	0.5	3.0
400, ad lib, control	69.3	2.9	27.8
SE	3.1	0.5	3.0
400, pair fed	63.6	2.5	33.9
SE	3.1	0.5	3.0

The incubation medium had 1.48 mg magnesium/100 ml, analyzed by atomic absorption spectroscopy, which could have been sufficient to allow for normal activity for the deficient neutrophils. To match the plasma magnesium concentration of deficient rats, DPBS medium with 0.72 mg magnesium/100 ml was used in duplicate assays for neutrophils from the rats consuming the 50 ug magnesium/gm diet. Comparisons using the deficient medium also were made for neutrophils from the control rats. No differences existed for neutrophils from deficient and control animals incubated in deficient medium compared to medium containing magnesium.

Experiment 2: Copper Deficiencies

The rats were similar before the dietary treatments in weight (116.0±1.8 gm), hemoglobin (13.0±0.2 gm/100 ml), hematocrit (36.7±0.4 percent), plasma copper (0.93±0.03 ug/ml), and ceruloplasmin (27.1±1.4 mU/ml). Significant differences then existed after the initiation of the treatments.

Three rats in the severely deficient, 0.5 ug copper/gm diet treatment group died after blood sampling, one at two weeks and the other two at four weeks. A rat on the 2.0 ug copper/gm diet died after the week six sampling period, and another severely deficient rat died before the end of the experiment. All deaths with the exception of the last, were attributed to internal bleeding after heart puncture.

Statistical analyses for missing observations and unbalanced data were used.

Nutritional Status

Plasma copper levels reflected the dietary levels of copper during the eight-week experiment ($P<0.001$). The lowest mean plasma copper concentration was for the rats on the 0.5 ug copper/gm diet as presented in Table 17. This was followed by mean plasma copper for rats on the 2.0 ug copper/gm diet and the 3.5 ug copper/gm diet. Highest mean levels were found in rats consuming the adequate 5.0 ug copper/gm diets, ad lib and with restricted intakes.

Table 17 also shows that with all three deficiencies, plasma copper decreased two weeks into the experiment. Later, mean plasma copper levels further dropped for the severely deficient rats, but slightly increased for rats on the marginally deficient diets.

By week eight, mean plasma copper was statistically similar for rats in the 3.5 ug copper/gm diet group and the two 5.0 ug copper/gm diet groups. Mean plasma copper remained significantly lower for rats on the 2.0 ug copper/gm diet and the 0.5 ug copper/gm diet.

Ceruloplasmin (Cp) activity was highly correlated with plasma copper at two weeks ($r=0.83$, $P<0.001$), at four weeks ($r=0.71$, $P<0.001$), and at eight weeks ($r=0.64$, $P<0.01$). Significant differences in Cp activity existed among the

Table 17. Least square mean plasma copper concentrations (ug/ml) for each copper dietary treatment at each sampling period (n=10).

Cu Diet ug/gm	Sampling Week Initial	2	4	8	Mean SE
0.5	0.97	0.10[c]	0.05[c]	0.06[c]	0.29[c] 0.08
2.0	1.03	0.38[c]	0.44[c]	0.58[c]	0.61[c] 0.06
3.5	0.94	0.69[a]	0.84[a]	1.11	0.89 0.06
5.0, ad lib, control	0.87	0.88	1.09	1.36	1.05 0.06
5.0, pair fed	0.86	0.89	1.03	1.17	0.99 0.06
Mean	0.93	0.59	0.69	0.86	
SE	0.03	0.03	0.03	0.06	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

diets, as presented in Table 18, and trends similar to those in plasma copper were seen throughout the experiment. The ferroxidase activity of Cp had no effect on catalase activity among the treatment groups.

The rats on the 0.5 ug copper/gm diet had significantly lower hemoglobin concentrations than the other treatment groups during the eight weeks of the experiment (Table 19). For the other dietary treatment groups, the hemoglobin increased throughout the study.

A more graded response was seen with the hematocrit measurements, with differences existing between the severely deficient, marginally deficient and copper-adequate animals (Table 20). Hematocrit increased more during the eight weeks for rats consuming the adequate copper diets, than for rats on the other diets.

Dietary copper significantly altered the growth of the rats. Those consuming the severely deficient, 0.5 ug copper/gm diet had the smallest overall mean weight (250.8$\pm$4.8 gm), which was matched by the pair-fed group (245.8$\pm$3.8 gm). Interestingly, rats on the 2.0 ug copper/gm diet had a mean weight equal to the control rats consuming 5.0 ug copper/gm ad lib (276.5$\pm$3.9 gm), while those on the 3.5 ug copper/gm diet had the highest mean weight (287.1$\pm$3.8 gm). The difference in the rats on the 3.5 ug copper/gm diet occurred after the fourth week of the experiment.

Table 18. Least square mean plasma ceruloplasmin activity (mU/ml) for each copper dietary treatment at each sampling period (n=10).

Cu Diet ug/gm	Sampling Week Initial	2	4	8	Mean SE
0.5	28.88	2.57[c]	1.66[c]	5.57[b]	9.67[c] 2.76
2.0	22.19	7.32[c]	10.82[c]	8.74[c]	12.26[c] 2.14
3.5	30.69	20.25	15.72[b]	34.28	25.24[b] 2.01
5.0, ad lib, control	27.63	30.88	34.00	36.76	32.32 2.01
5.0, pair fed	26.06	36.69	25.38	44.25	33.10 2.01
Mean	27.09	19.54	17.52	25.92	
SE	1.38	1.38	2.25	2.60	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

Table 19. Least square mean hemoglobin (gm/100 ml) for each copper dietary treatment at each sampling period (n=10).

Cu Diet ug/gm	Sampling Week Initial	2	4	8	Mean SE
0.5	12.6	11.7[c]	11.5[c]	13.5[b]	12.3[c] 0.3
2.0	12.8	13.3	15.2	16.1	14.3 0.3
3.5	13.2	13.5	15.6	15.3[a]	14.4 0.2
5.0, ad lib, control	13.0	13.7	16.0	16.9	14.9 0.2
5.0, pair fed	13.4	15.1[b]	16.1	17.5	15.5 0.2
Mean	13.0	13.5	14.9	15.9	
SE	0.2	0.2	0.2	0.3	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

Table 20. Least square mean hematocrit (%) for each copper dietary treatment at each sampling period (n=10).

Cu Diet ug/gm	Sampling Week Initial	2	4	8	Mean SE
0.5	36.8	33.3[c]	32.6[c]	37.9[b]	35.2[c] 0.7
2.0	35.4	38.0	39.3[c]	40.7[b]	38.3[c] 0.6
3.5	36.6	39.0	43.0	42.0[a]	40.2 0.6
5.0, ad lib, control	37.0	38.4	43.5	45.8	41.2 0.6
5.0, pair fed	37.8	41.3[a]	44.2	46.1	42.4 0.6
Mean	36.7	38.0	40.5	42.5	
SE	0.4	0.4	0.4	0.7	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

Total weight gains did not significantly differ among treatment groups until after week four (Table 21). Then, the severely deficient rats and their pair-fed mates had similar total weight gains. These were significantly lower than weight gains for rats in the other treatment groups. Rats on the 3.5 ug copper/gm diet gained the most weight.

Feed efficiency, also shown in Table 21, differed among the treatment groups, reflecting the trends observed in weight gained. At four weeks, feed efficiencies were similar for all treatment groups, as were weight gains.

After eight weeks, rats on the 0.5 ug copper/gm diet had a low mean feed efficiency. Their pair-fed mates on the 5.0 ug copper/gm diet had a slightly higher mean feed efficiency. The other rats on the marginal diets with 2.0 ug copper and 3.5 ug copper/gm were similar to those on the control, ad lib 5.0 ug copper/gm diet. Only those on the severely deficient diet were statistically different from the controls. For all rats, feed efficiency was higher at three weeks than at eight weeks.

Actual mean liver weights were not different among rats in the treatment groups. Yet, when expressed as a percent of total body weight as in Table 22, livers from the severely deficient rats were significantly larger than those from animals in the other groups, including the pair-fed rats. Liver weight as a percent of body weight was negatively correlated with plasma copper at four weeks ($r=-0.43$, $P<0.05$) and at eight weeks ($r=-0.74$, $P<0.001$).

Table 21. Least square mean weight gain (gm), and feed efficiency (gm weight gain/gm feed intake) for each copper treatment at each sampling period (n=5).

	Sampling Week 4		8		Mean SE	
Cu Diet ug/gm	weight gain	feed eff.	weight gain	feed eff.	weight gain	feed eff.
0.5	161.9	0.30	202.7[c]	0.21	182.3[c] 9.1	0.25[a] 0.01
2.0	180.0	0.32	265.6	0.21	222.8 8.0	0.27 0.01
3.5	187.8	0.32	301.5	0.25	244.7 7.5	0.28 0.01
5.0, ad lib, control	184.3	0.32	276.4	0.23	230.4 7.5	0.27 0.01
5.0, pair fed	162.7	0.31	208.1[c]	0.21	185.4[c] 7.5	0.26 0.01
Mean	175.4	0.31	250.9	0.22		
SE	4.9	0.00	5.2	0.00		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

Table 22. Least square mean liver weights (gm) as percent of total body weight (gm) and liver copper concentration (ug/gm liver, wet weight) for each copper dietary treatment at each sampling period (n=5).

Cu Diet ug/gm	Sampling Week 4		Sampling Week 8		Mean SE	
	weight	Cu	weight	Cu	weight	Cu
0.5	5.40	0.45	5.45	0.42	5.43[a] 0.23	0.43[c] 0.20
2.0	4.96	2.07	4.67	2.32	4.82 0.20	2.20[c] 0.18
3.5	4.84	3.12	4.23	3.19	4.54 0.19	3.16 0.17
5.0, ad lib, control	4.84	3.14	4.23	3.58	4.53 0.19	3.36 0.17
5.0, pair fed	4.84	2.85	4.25	2.93	4.54 0.19	2.89 0.17
Mean	4.98	2.32	4.57	2.49		
SE	0.12	0.11	0.13	0.11		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

Table 22 also shows that liver copper was significantly altered by dietary copper. Plasma copper highly correlated with liver copper stores at four weeks ($r=0.76$, $P<0.001$) and at eight weeks ($r=0.87$, $P<0.001$). Similarly, Cp highly correlated with liver copper at four weeks ($r=0.66$, $P<0.001$) and at eight weeks ($r=0.62$, $P<0.01$).

The severely deficient diet depleted four and eight week liver copper concentrations to 13 percent of the control value. The 2.0 ug copper/gm diet also significantly reduced liver copper to 65 percent of the control. The other marginal diet of 3.5 ug copper/gm was similar to the control. Restriction of feed intake also affected liver copper which was 86 percent of the ad lib control.

Lymphoid Organs

The spleen and thymus weights, like liver weights, were expressed as a percent of total body weight for comparisons (Table 23). At four weeks, the mean weight of spleens from all rats was 0.23 ± 0.01 percent of total body weight, and at eight weeks it was 0.21 ± 0.01 percent of total body weight. Dietary treatment had no effect. On the other hand, the thymuses were affected by dietary copper. The severely deficient rats had thymuses that were a smaller percentage of total body weight compared to controls, while others were similar to the controls. The percentage for all rats at four weeks, 0.20 ± 0.01 percent, dropped to 0.15 ± 0.01 percent,

Table 23. Least square mean spleen and thymus weights (gm) as percent of total body weight (gm) for each copper dietary treatment at each sampling period (n=5).

Cu Diet ug/gm	Sampling Week 4		8		Mean SE	
	spleen	thymus	spleen	thymus	spleen	thymus
0.5	0.21	0.16	0.23	0.10	0.22 0.01	0.13[b] 0.02
2.0	0.25	0.22	0.22	0.17	0.23 0.01	0.19 0.01
3.5	0.23	0.24	0.19	0.18	0.21 0.01	0.21 0.01
5.0, ad lib, control	0.23	0.22	0.20	0.16	0.21 0.01	0.19 0.01
5.0, pair fed	0.24	0.19	0.21	0.16	0.22 0.01	0.17 0.01
Mean	0.23	0.20	0.21	0.15		
SE	0.01	0.01	0.01	0.01		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

indicating a greater change in body weight than in thymus weight during the eight-week time period.

Cellular Immunity

Total and differential white blood cell counts were not altered by dietary copper as shown in Table 24. Some statistically significant changes in the differential percentages occurred over time. Mean lymphocyte counts decreased from 6.5 x 10^3/ul at four weeks to 5.8 x 10^3/ul at eight weeks with concomitant increases in all other leukocytes.

The percentages of T- and B-cells also were not changed by copper deficiency. Of the total lymphocytes, 68 to 77$\pm$3 percent were T cells, while 23 to 27$\pm$2 percent were B cells.

When mean comparisons were conducted on the lymphocyte stimulation data, large variation contributed to no differences according to diet, although some general trends appeared. As indicated in Table 25, all mean lymphocyte stimulation indexes for rats on the 0.5 ug copper/gm diet were lower than those of control animals. The PHA SI was 13.6 percent of the control, Con A SI was 37.6 percent of the control, and PWM SI was 45.6 percent of the control.

The PHA SI and PWM SI for rats on the 2.0 ug copper/gm diet also were lower than the control, while the Con A SI was higher than the control. Rats on the 3.5 ug copper/gm diet had a Con A SI that was similar to those on the 2.0 ug copper/gm diet.

Table 24. Least square mean total white blood cell counts (x 10^3/ul) and differentials for each copper dietary treatment for both sampling periods (n=10).

Cu Diet ug/gm	Total WBC	Percent Baso	Percent Eos	Percent Neut	Percent Lymph	Percent Mono
0.5	6.1	0.0	0.9	11.4	84.8	2.7
SE	1.2	0.2	0.5	2.5	2.7	0.7
2.0	7.1	0.0	1.0	9.5	86.8	2.4
SE	0.8	0.1	0.4	1.9	2.1	0.5
3.5	6.6	0.1	1.1	7.7	88.6	2.5
SE	0.7	0.1	0.4	1.8	2.0	0.5
5.0, ad lib, control	7.2	0.1	1.2	8.6	87.6	2.4
SE	0.7	0.1	0.4	1.8	2.0	0.5
5.0, pair fed	7.1	0.5	1.1	6.8	88.9	2.6
SE	0.7	0.1	0.4	1.8	1.9	0.5

differentials (x 10^3/ul) based on percent of total WBC:

Cu Diet ug/gm	Baso	Eos	Neut	Lymph	Mono
0.5	0.0	0.1	0.7	5.2	0.2
2.0	0.0	0.1	0.7	6.2	0.2
3.5	0.0	0.1	0.5	5.9	0.2
5.0, ad lib, control	0.0	0.1	0.6	6.3	0.2
5.0, pair fed	0.0	0.1	0.5	6.3	0.2

Table 25. Least square mean lymphocyte stimulation responses for each copper dietary treatment for both sampling periods (n=10).

Cu Diet ug/gm	PHA		Con A		PWM	
	CPM*	SI**	CPM*	SI**	CPM*	SI**
0.5	232	1.5	2,549	15.7	811	5.7
SE	1,176	4.1	5,153	26.7	920	3.1
2.0	873	5.2	13,594	72.8	2,311	9.7
SE	885	3.1	3,880	20.1	693	2.4
3.5	896	3.1	16,782	72.2	2,939	10.9
SE	828	2.9	3,629	18.8	648	2.2
5.0, ad lib, control	3,398	11.0	11,716	41.7	3,581	12.5
SE	828	2.9	3,629	18.8	648	2.2
5.0, pair fed	1,411	7.3	11,214	62.9	2,599	12.7
SE	828	2.9	3,629	18.8	648	2.2

*, Counts per minute
**, Stimulation index

Regression analysis provided greater insight as to how dietary copper affected lymphocyte proliferation. At four weeks, PHA CPM fit a linear and quadratic curve with plasma copper concentrations as the independent variable ($P<0.05$). The PHA SI regressions also were significant ($P<0.05$).

Humoral Immunity

Dietary copper deficiencies did not significantly affect total plasma protein, nor did they change albumin and globulin distributions (Table 26). There were small, but statistically significant, increases in the beta-and gamma globulin concentrations between four and eight weeks. These had no overall effects on the albumin/globulin ratio.

For all rats, IgM and IgG levels increased between the four- and eight-week sampling periods. Table 27 shows that the severely deficient rats had a mean IgM concentration which was 38 percent that of all other treatment groups. Rats on the 2.0 ug copper/gm diet had a higher IgG concentration than all others. No differences existed among the other diets.

Specific antibody response to heterologous RBCs was variable among the dietary treatments (Table 28). Regression analysis was used to test whether copper status had an effect on antibody response. Plasma copper was the independent variable and specific antibody titer the dependent variable. At four weeks, no linear or quadratic relationship existed. At eight weeks, a quadratic

Table 26. Least square mean total plasma protein, albumin and globulins (gm/100 ml) for each copper dietary treatment for both sampling periods (n=10).

Cu Diet ug/gm	Total Protein	Albumin	Globulins				A/G Ratio
			A1	A2	Beta	Gamma	
0.5	11.18	5.53	1.18	0.98	2.71	0.33	1.08
SE	1.11	0.66	0.18	0.11	0.27	0.05	0.06
2.0	10.52	5.25	1.58	0.95	2.46	0.30	1.01
SE	0.80	0.48	0.14	0.08	0.21	0.04	0.05
3.5	9.33	4.87	1.32	0.79	2.06	0.25	1.01
SE	0.75	0.46	0.14	0.08	0.20	0.04	0.05
5.0, ad lib, control	9.62	4.92	1.47	0.85	2.13	0.29	1.06
SE	0.75	0.45	0.13	0.08	0.20	0.04	0.05
5.0, pair fed	9.61	4.92	1.51	0.79	2.16	0.28	1.03
SE	0.76	0.45	0.13	0.08	0.20	0.04	0.05

Table 27. Least square mean IgM and IgG (mg/ml) for each copper dietary treatment at each sampling period (n=5).

Cu Diet ug/gm	Sampling Week 4 IgM	4 IgG	8 IgM	8 IgG	Mean SE IgM	Mean SE IgG
0.5	0.46	9.48	0.74	12.78	0.60[c]	11.13
					0.20	1.93
2.0	1.17	11.59	1.72	18.84	1.45	15.21[a]
					0.16	1.52
3.5	1.42	6.82	1.55	11.76	1.49	9.29
					0.15	1.41
5.0, ad lib, control	1.48	10.20	1.66	10.53	1.57	10.36
					0.16	1.43
5.0, pair fed	1.42	10.68	2.16	13.68	1.79	12.18
					0.15	1.41
Mean	1.19	9.75	1.57	13.52		
SE	0.10	0.91	0.11	1.04		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

Table 28. Least square mean specific antibody titer and log titer for each copper dietary treatment at each sampling period (n=5).

	Sampling Week 4		8		Mean SE	
Cu Diet ug/gm	Titer	Log Titer	Titer	Log Titer	Titer	Log Titer
0.5	22	6	33	13	27 7	9 2
2.0	23	9	7	3	15 6	6 2
3.5	16	7	18	8	17 5	8 1
5.0, ad lib, control	10	5	6	3	8 5	4 1
5.0, pair fed	15	4	18	6	16 6	5 2
Mean	17	6	16	7		
SE	3	1	4	1		

relationship was significant ($P<0.01$). Similar results were observed when specific antibody was expressed as the log of the reciprocal titer ($P>0.01$ at eight weeks).

Plasma copper concentrations varied within the marginal, 2.0 ug copper/gm diet treatment (0.25 to 0.98 ug/ml), and within the 3.5 ug copper/gm treatment (0.65 to 1.66 ug/ml). In general, it appeared that specific antibody response was less at marginal plasma copper concentrations compared to higher and very low plasma copper concentrations.

Neutrophil Function

Table 29 shows that neutrophil phagocytosis of E. coli and intracellular killing was statistically similar for all rats, regardless of diet. Yet, more than half of the neutrophils from the severely deficient rats (0.5 ug copper/gm diet) ingested E. coli, while less than half of the neutrophils from rats in the other treatments ingested the bacteria. At week four, 50 to 60 percent of the neutrophils that ingested E. coli, also showed bactericidal activity. Then at eight weeks, killing was observed in more than 90 percent of neutrophils with ingested E. coli.

The neutrophil cupro-enzyme, SOD, was not altered by the treatments (Table 30), nor by changes in plasma copper or Cp. The ferroxidase activity of Cp also appeared not to affect neutrophil MPO activity. Neutrophil phagocytosis and killing did not correlate with SOD activity at week four. On

Table 29. Least square mean neutrophil phagocytosis and killing (%) for each copper dietary treatment for both sampling periods (n=10).

Cu Diet ug/gm	% neutrophils without bacteria	with bacteria, not killed	with bacteria, killed
0.5	44.7	10.5	44.8
SE	6.3	2.8	6.2
2.0	56.4	9.6	34.0
SE	4.7	2.1	4.7
3.5	62.9	11.8	25.0
SE	4.4	2.0	4.4
5.0, ad lib, control	57.5	12.6	29.9
SE	4.4	2.0	4.4
5.0, pair fed	63.2	8.4	28.4
SE	4.4	2.0	4.4

Table 30. Least square mean neutrophil myeloperoxidase (MPO) and superoxide dismutase (SOD) in U x 10^{-7}/neutrophil for each copper dietary treatment at each sampling period (n=5).

Cu Diet ug/gm	Sampling Week 4		8		Mean SE	
	MPO	SOD	MPO	SOD	MPO	SOD
0.5	0.32	114.1	1.70	149.7	1.01 0.29	131.9 29.1
2.0	0.74	129.8	1.08	117.6	0.91 0.23	123.7 22.5
3.5	0.57	102.7	1.85	139.7	1.21 0.22	121.2 21.3
5.0, ad lib, control	0.78	131.2	1.60	119.8	1.19 0.22	125.5 21.3
5.0, pair fed	0.52	205.3	1.15	152.6	0.84 0.22	179.0 21.3
Mean	0.59	136.6	1.48	135.9		
SE	0.14	13.8	0.16	15.6		

the other hand, at week four, MPO activity and neutrophil ingestion and killing were significantly correlated ($r=0.52$, $P<0.01$). At week eight, the correlation was not as strong ($r=0.40$, $P<0.07$). SOD activity correlated with neutrophil ingestion and killing at week eight ($r=0.44$, $P<0.05$).

Experiment 3: Magnesium and Copper Deficiencies

The rats were similar before the dietary treatments in weight (116.9±2.0 gm), hemoglobin (12.5±0.2 gm/100 ml), hematocrit (35.9±0.5 percent), plasma magnesium (1.80±0.02 mg/100 ml), and plasma copper (1.15±0.03 ug/ml). Significant differences in all nutritional parameters then existed after the initiation of the treatments.

There were four deaths caused by internal bleeding after heart puncture during the experiment. Two rats on the severely deficient diet, one rat on the 280 ug magnesium and 2.0 ug copper/gm diet, and one on the 160 ug magnesium and 3.5 ug copper/gm diet, died after the week two sampling period. Statistical analyses for missing observations and unbalanced data were used.

Clinical signs of magnesium deficiency were apparent in rats receiving the 50 ug magnesium and 0.5 ug copper/gm dietary treatment. These included ulcerative lesions about the head and neck, a coarse hair coat, and skin necrosis. Hyperirritability also was noticeable, and seizure activity was observed in one rat during the fifth week of the experiment.

Nutritional Status

Severe and moderately deficient levels of dietary magnesium affected plasma magnesium during the eight-week experiment as shown in Table 31. Rats receiving 50 ug magnesium/gm diet had significantly lower plasma magnesium concentrations after initiation of the treatments than all other treatment groups ($P<0.001$). Rats receiving moderately deficient levels of magnesium (160 ug/gm) had low mean plasma magnesium regardless of dietary copper concentration. Mean plasma magnesium for both groups were significantly lower than controls.

Throughout the experiment, the rats consuming less copper had slightly lower plasma magnesium concentrations than rats consuming more copper. Rats consuming the diets with 280 ug magnesium/gm had plasma magnesium levels similar to control rats consuming 400 ug magnesium/gm.

Bone magnesium also was significantly affected by severe (50 ug/gm) and moderate (160 ug/gm) dietary magnesium deficiencies, regardless of dietary copper levels (Table 32). The lowest mean bone magnesium concentration was found in the 50 ug magnesium and 0.5 ug copper/gm diet group, followed by the 160 ug magnesium/gm diets with 2.0 ug copper/gm and 3.5 ug copper/gm. Rats consuming 280 ug magnesium/gm had bone magnesium concentrations similar to the control animals. The controls consumed 400 ug magnesium/gm, and had a mean bone magnesium concentration of 4.50 mg/gm bone. The amount of magnesium in the bone also

Table 31. Least square mean plasma magnesium (mg/100 ml) for each combined magnesium and copper dietary treatment at each sampling period (n=10).

Diet ug/gm	Sampling Week Initial	2	4	8	Mean SE
50 Mg 0.5 Cu	1.87	0.44[c]	0.34[c]	0.44[c]	0.77[c] 0.04
160 Mg 2.0 Cu	2.03	1.15[c]	1.17[b]	1.24[c]	1.40[c] 0.03
280 Mg 2.0 Cu	1.74	1.51	1.61	1.60	1.61 0.03
160 Mg 3.5 Cu	1.70	1.33[b]	1.57	1.29[b]	1.47[c] 0.03
280 Mg 3.5 Cu	1.72	1.54	1.79	1.67	1.68 0.03
400 Mg 5.0 Cu	1.75	1.56	1.56	1.65	1.63 0.03
Mean	1.80	1.25	1.34	1.31	
SE	0.02	0.02	0.04	0.04	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

Table 32. Least square mean bone magnesium (mg/gm bone, dry weight) for each combined magnesium and copper dietary treatment at each sampling period (n=5).

Diet ug/gm	Sampling Week 4	Sampling Week 8	Mean SE
50 Mg 0.5 Cu	2.00	1.39	1.69[c] 0.16
160 Mg 2.0 Cu	3.40	3.97	3.69[c] 0.15
280 Mg 2.0 Cu	4.35	4.78	4.57 0.15
160 Mg 3.5 Cu	3.78	3.79	3.78[b] 0.15
280 Mg 3.5 Cu	4.14	4.41	4.28 0.15
400 Mg 5.0 Cu	4.56	4.44	4.50 0.15
Mean	3.71	3.80	
SE	0.09	0.09	

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

highly correlated with plasma magnesium at four weeks ($r=0.74$, $P<0.001$) and at eight weeks ($r=0.92$, $P<0.001$).

All levels of dietary copper affected plasma copper concentrations throughout the experiment ($P<0.001$). Dietary magnesium appeared to have no influence on the plasma copper concentration. Plasma copper concentrations reflected dietary copper levels as shown in Table 33. Differences were observed at week two and remained throughout the eight weeks.

Cp followed a similar trend, as presented in Table 34, and highly correlated with plasma copper at week two ($r=0.92$, $P<0.001$), at week four ($r=0.86$, $P<0.001$), and at week eight ($r=0.94$, $P<0.001$). The mean Cp during the whole experiment was similar for the diets with 0.5 ug copper/gm and 2.0 ug copper/gm. However, Cp activity initially was higher, then dramatically decreased at week two in the severely deficient rats and remained extremely low by the eight-week sampling period. On the other hand, Cp activity for the rats consuming 2.0 ug copper/gm began to recover after the initial drop at week two.

The ferroxidase activity of Cp did not appear to immediately affect catalase. By eight weeks, however, Cp activity and catalase activity were significantly correlated ($r=0.53$, $P<0.01$). Rats on the severely deficient diet had the lowest mean catalase activity. Those consuming the 280 ug magnesium and 3.5 ug copper/gm diet were similar to the control rats, with the highest catalase activity.

Table 33. Least square mean plasma copper concentrations (ug/ml) for each combined magnesium and copper dietary treatment at each sampling period (n=10).

Diet ug/gm	Sampling Week Initial	2	4	8	Mean SE
50 Mg 0.5 Cu	1.15	0.25[c]	0.25[c]	0.19[c]	0.46[c] 0.05
160 Mg 2.0 Cu	1.15	0.42[c]	0.52[c]	0.79[c]	0.72[c] 0.05
280 Mg 2.0 Cu	1.14	0.35[c]	0.57[c]	0.66[c]	0.68[c] 0.05
160 Mg 3.5 Cu	1.12	0.84[b]	1.10[a]	1.41	1.12[b] 0.05
280 Mg 3.5 Cu	1.16	0.81[b]	1.07[b]	1.28[a]	1.08[c] 0.05
400 Mg 5.0 Cu	1.19	1.07	1.44	1.59	1.32 0.05
Mean	1.15	0.62	0.83	0.99	
SE	0.03	0.03	0.04	0.04	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

Table 34. Least square mean plasma ceruloplasmin activity (mU/ml) for each combined magnesium and copper dietary treatment at each sampling period (n=10).

Diet ug/gm	Sampling Week Initial	2	4	8	Mean SE
50 Mg 5.0 Cu	32.75	2.69[c]	5.22[c]	0.46[c]	10.28[c] 2.62
160 Mg 2.0 Cu	16.00[c]	4.38[c]	8.32[c]	18.81[c]	11.88[c] 2.34
280 Mg 2.0 Cu	15.00[c]	3.82[c]	9.79[c]	13.86[c]	10.62[c] 2.48
160 Mg 3.5 Cu	20.63[c]	29.25	23.94[b]	50.80	31.16[c] 2.48
280 Mg 3.5 Cu	34.25	25.25[a]	38.25	47.88	36.41[b] 2.34
400 Mg 5.0 Cu	36.63	34.57	42.10	56.03	42.33 2.34
Mean	25.88	16.66	21.27	31.31	
SE	1.26	1.26	2.05	2.14	

a, different from control within each week (P<0.05)
b, different from control within each week (P<0.01)
c, different from control within each week (P<0.001)

Liver copper content also reflected dietary copper levels. For each level of dietary copper, liver copper content was significantly different from all others. Table 35 shows that all dietary treatments were significantly different from the control. Dietary magnesium did not appear to influence liver copper.

Rats consuming 0.5 ug copper/gm had liver stores depleted to 13 percent of the control values. The diets with 2.0 ug copper/gm reduced liver copper to approximately 55 percent of the control. Those with 3.5 ug copper/gm lowered liver copper to an average of 83 percent of the control. The amount of copper in the liver also was highly correlated at four weeks with plasma copper concentration ($r=0.93$, $P<0.001$) and Cp ($r=0.80$, $P<0.001$). High correlations also were found at eight weeks between liver copper and plasma copper ($r=0.90$, $P<0.001$) and Cp ($r=0.82$, $P<0.001$).

In contrast to the changes observed in liver copper with various dietary copper levels, only the diet with 0.5 ug copper/gm altered liver weight when expressed as a percent of total body weight (Table 35). Livers were significantly enlarged by the severely deficient dietary treatment, and were bleached in color.

By the end of the experiment hemoglobin and hematocrit were significantly lower in the severely deficient rats consuming the 50 ug magnesium and 0.5 ug copper/gm diet than for all other rats (Tables 36 and 37). There were no differences between the marginal dietary deficiencies and

Table 35. Least square mean liver weights (gm) as percent of total body weight (gm), and liver copper concentration (ug/gm liver, wet weight) for each combined magnesium and copper dietary treatment at each sampling period (n=5).

Diet ug/gm	Sampling Week 4		Sampling Week 8		Mean SE	
	weight	Cu	weight	Cu	weight	Cu
50 Mg 0.5 Cu	5.52	0.71	6.59	0.25	6.05[c] 0.16	0.48[c] 0.20
160 Mg 2.0 Cu	5.12	1.97	4.49	2.20	4.80 0.15	2.08[c] 0.18
280 Mg 2.0 Cu	5.07	2.16	4.40	1.89	4.73 0.16	2.02[c] 0.19
160 Mg 3.5 Cu	5.05	3.03	4.01	2.91	4.53 0.16	2.97[b] 0.19
280 Mg 3.5 Cu	4.74	3.27	4.41	3.14	4.57 0.15	3.20[a] 0.18
400 Mg 5.0 Mg	4.65	4.02	4.35	3.46	4.50 0.15	3.74 0.18
Mean	5.02	2.53	4.71	2.31		
SE	0.09	0.11	0.09	0.11		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

Table 36. Least square mean hemoglobin (gm/100 ml) for each combined magnesium and copper dietary treatment at each sampling period (n=10).

Diet ug/gm	Sampling Week Initial	2	4	8	Mean SE
50 Mg 0.5 Cu	11.5	13.0[a]	11.7	8.5[c]	11.2[c] 0.4
160 Mg 2.0 Cu	12.2	13.9	14.4	15.0	13.8 0.3
280 Mg 2.0 Cu	12.9	14.2	13.9	14.7	13.9 0.4
160 Mg 3.5 Cu	12.5	14.0	13.4	15.1	13.7 0.4
280 Mg 3.5 Cu	12.9	14.5	14.2	15.5	14.3 0.3
400 Mg 5.0 Cu	12.8	14.5	13.9	15.7	14.2 0.3
Mean	12.5	14.0	13.6	14.1	
SE	0.2	0.2	0.3	0.3	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

Table 37. Least square mean hematocrit (%) for each combined magnesium and copper dietary treatment at each sampling period (n=10).

Diet ug/gm	Sampling Week Initial	2	4	8	Mean SE
50 Mg 0.5 Cu	36.0	37.0	34.5[b]	28.9[c]	34.1[c] 0.8
160 Mg 2.0 Cu	36.4	40.2	42.4	45.0	40.0 0.8
280 Mg 2.0 Cu	36.9	40.3	41.1	44.0	40.6 0.8
160 Mg 3.5 Cu	36.0	40.4	38.6	45.3	40.1 0.8
280 Mg 3.5 Cu	33.3	40.4	40.8	47.3	40.4 0.8
400 Mg 5.0 Cu	36.7	39.5	42.7	45.1	41.0 0.8
Mean	35.9	39.6	40.0	52.6	
SE	0.5	0.5	0.7	0.8	

a, different from control within each week ($P<0.05$)
b, different from control within each week ($P<0.01$)
c, different from control within each week ($P<0.001$)

the control diet throughout the experiment. For those rats, hematocrit increased with time, as did hemoglobin to a lesser extent, while decreases were observed in the 50 ug magnesium and 0.5 ug copper/gm diet group.

The combined magnesium and copper marginal deficiencies did not significantly affect growth. Weight gain was similar among all groups except for those on the severely deficient 50 ug magnesium and 0.5 ug copper/gm diet (Table 38). The difference in weight occurred early in the experiment and continued to the end. The mean weight gains at four and eight weeks for the severely deficient rats were approximately 60 percent that for control animals.

With the exception of the severely deficient rats, feed efficiency was similar for all dietary treatments, also shown in Table 38. Mean feed efficiency during the eight-week experiment was significantly lower for the rats consuming the 50 ug magnesium and 0.5 ug copper/gm diet, than for rats consuming marginal or adequate levels of both nutrients.

Lymphoid Organs

Both the spleen and thymus were affected by a severe combined dietary deficiency of magnesium and copper. When organ weight was expressed as a percent of total body weight, spleens were significantly enlarged by the 50 ug magnesium and 0.5 ug copper/gm diet, and thymuses were much smaller than those from animals on the other dietary

Table 38. Least square mean weight gain (gm), and feed efficiency (gm weight gain/gm feed intake) for each combined magnesium and copper dietary treatment at each sampling period (n=5).

	Sampling Week 4		8		Mean SE	
Diet ug/gm	weight gain	feed eff.	weight gain	feed eff.	weight gain	feed eff.
50 Mg	118.1	0.24	171.7	0.17	144.9[c]	0.20[c]
0.5 Cu					10.8	0.01
160 Mg	180.4	0.31	266.9	0.23	223.7	0.27
2.0 Cu					9.7	0.01
280 Mg	173.2	0.31	264.2	0.23	218.7	0.27
2.0 Cu					10.3	0.01
160 Mg	162.4	0.30	272.2	0.23	217.3	0.27
3.5 Cu					10.3	0.01
280 Mg	178.7	0.32	271.8	0.23	225.3	0.28
3.5 Cu					9.7	0.01
400 Mg	191.7	0.34	281.2	0.23	236.5	0.29
5.0 Cu					9.7	0.01
Mean	167.4	0.31	254.7	0.22		
SE	5.7	0.01	5.9	0.01		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

treatments (Table 39). As total body weight increased from week four to eight, the percentage decreased for both spleen and thymus weight.

Cellular Immunity

Total white blood cell counts were altered by the severely deficient, 50 ug magnesium and 0.5 ug copper/gm diet as shown in Table 40. The mean total WBC count at week four for rats on that diet was twice the amount for rats on the control diet (14.0×10^3/ul and 7.0×10^3/ul, respectively). There was a significant increase of neutrophils. By week eight, however, the neutrophil count decreased, and the total WBC count for the severely deficient rats dropped to a level that was not significantly different from the controls (7.3×10^3/ul and 8.6×10^3/ul, respectively).

At four weeks total WBC count was negatively correlated with plasma magnesium ($r=-0.66$, $P<0.001$), and also to plasma copper ($r=-0.57$, $P<0.001$). Then, at eight weeks, mean total WBC counts, as well as the differentials, were similar for all dietary treatments.

The percentage of B cells was not significantly changed by combined dietary magnesium and copper deficiencies, as shown in Table 41. The mean percentage dropped from 26 ± 2 percent at four weeks to 20 ± 2 percent at eight weeks.

A similar decrease over time was observed with the percentage of T cells (74 ± 2 percent at four weeks to

Table 39. Least square mean spleen and thymus weights (gm) as percent of total body weight (gm) for each combined magnesium and copper dietary treatment at each sampling period (n=5).

Diet ug/gm	Sampling Week 4		8		Mean SE	
	spleen	thymus	spleen	thymus	spleen	thymus
50 Mg 0.5 Cu	0.43[c]	0.20	0.31[c]	0.10	0.37[c] 0.01	0.15[b] 0.01
160 Mg 2.0 Cu	0.26	0.25	0.21	0.17	0.24 0.01	0.21 0.01
280 Mg 2.0 Cu	0.22	0.23	0.21	0.14	0.21 0.01	0.18 0.01
160 Mg 3.5 Cu	0.23	0.24	0.20	0.15	0.22 0.01	0.20 0.01
280 Mg 3.5 Cu	0.26	0.23	0.21	0.16	0.24 0.01	0.19 0.01
400 Mg 5.0 Cu	0.24	0.24	0.21	0.16	0.22 0.01	0.20 0.01
Mean	0.27	0.23	0.22	0.15		
SE	0.01	0.01	0.01	0.01		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

Table 40. Least square mean total white blood cell counts (x 10^3/ul) and differentials for each combined magnesium and copper dietary treatment for both sampling periods (n=10).

Diet ug/gm	Total WBC	Percent Baso	Percent Eos	Percent Neut	Percent Lymph	Percent Mono
50 Mg						
0.5 Cu	10.7[b]	0.3[a]	2.8	22.8[c]	71.3[c]	3.5
SE	0.7	0.3	0.5	2.2	2.7	0.6
160 Mg						
2.0 Cu	8.8	0.3[a]	1.6	8.3	87.6	1.4[a]
SE	0.7	0.3	0.4	2.0	2.5	0.5
280 Mg						
2.0 Cu	7.2	0.1[b]	1.1	9.4	87.1	2.3
SE	0.7	0.3	0.5	2.1	2.5	0.5
160 Mg						
3.5 Cu	6.5	0.9	0.8	11.0	85.4	2.0
SE	0.7	0.3	0.5	2.1	2.5	0.5
280 Mg						
3.5 Cu	7.3	0.4[a]	1.3	11.6	83.2	3.2
SE	0.7	0.3	0.4	1.9	2.4	0.5
400 Mg						
5.0 Cu	7.8	1.1	1.9	9.7	84.2	3.0
SE	0.7	0.3	0.4	1.9	2.4	0.5

a, ($P<0.05$); b, ($P<0.01$); c, ($P<0.001$)

differentials (x 10^3/ul) based on percent of total WBC:

Diet ug/gm	Baso	Eos	Neut	Lymph	Mono
50 Mg 0.5 Cu	0.0	0.3	2.4	7.6	0.4
160 Mg 2.0 Cu	0.0	0.1	0.7	7.7	0.1
280 Mg 2.0 Cu	0.0	0.1	0.7	6.3	0.2
160 Mg 3.5 Cu	0.1	0.1	0.7	5.6	0.1
280 Mg 3.5 Cu	0.0	0.1	0.8	6.1	0.2
400 Mg 5.0 Cu	0.1	0.1	0.8	6.6	0.2

Table 41. Least square mean B cells (%) and T cells (%) for each combined magnesium and copper dietary treatment at each sampling period (n=5).

Diet ug/gm	Sampling Week 4		8		Mean SE	
	B cell	T cell	B cell	T cell	B cell	T cell
50 Mg 0.5 Cu	26	87	18	68	22 3	78[b] 4
160 Mg 2.0 Cu	20	83	16	61	18 3	72[a] 3
280 Mg 2.0 Cu	23	70	19	60	21 3	65 3
160 Mg 3.5 Cu	32	73	14	62	23 3	67 3
280 Mg 3.5 Cu	25	69	25	61	25 3	65 3
400 Mg 5.0 Cu	31	64	25	59	28 3	62 3
Mean	26	74	20	62		
SE	2	2	2	2		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

62±2 percent at eight weeks). Table 41 also shows that diet had an influence on mean T cell percentages. Rats on the 50 ug magnesium and 0.5 ug copper/gm diet and the 160 ug magnesium and 2.0 ug copper/gm diet had an elevated T cell percentage when compared to rats on the other dietary treatments. Furthermore, T cell percentage was increased by low dietary magnesium (160 ug/gm), especially when combined with low dietary copper (2.0 ug/gm).

Data for lymphocyte stimulation was available only on the eight-week samples. The four-week samples could not be used due to contamination. Lymphocyte proliferation was not statistically different among dietary treatments with PHA and Con A stimulation (Table 42).

Although large variation existed in the data, mean PHA CPM for lymphocytes from the rats consuming the severely deficient, 50 ug magnesium and 0.5 ug copper/gm diet were 10 percent of the control, and the mean SI was 44 percent of the control. In addition, the mean CPM for lymphocytes from rats on the marginally deficient, 160 ug magnesium and 2.0 ug copper/gm diet were 68 percent of the control, and the mean SI was 87 percent of the control.

Significant differences among dietary treatments existed when PWM was used to stimulate lymphocyte proliferation. The mean CPM was lowered by the severely deficient levels of magnesium (50 ug/gm) and copper (0.5 ug/gm), the moderately deficient level of copper (2.0 ug/gm), and the mildly deficient level of magnesium

Table 42. Least square mean lymphocyte stimulation responses for each combined magnesium and copper dietary treatment for the week eight sampling period (n=5).

Diet ug/gm	PHA		Con A		PWM	
	CPM*	SI**	CPM*	SI**	CPM*	SI**
50 Mg 0.5 Cu	343	3.2	21,061	105.9	6,418[b]	38.6
SE	1,469	3.3	7,378	34.4	3,496	12.1
160 Mg 2.0 Cu	2,230	6.2	22,748	73.0	8,221[b]	22.5
SE	1,287	2.9	6,467	30.2	3,065	10.6
280 Mg 2.0 Cu	3,189	8.9	20,675	101.7	8,374[b]	38.2
SE	1,469	3.3	7,378	34.4	3,496	12.1
160 Mg 3.5 Cu	3,265	6.8	29,676	89.2	19,766	49.4
SE	1,469	3.3	7,378	34.4	3,496	12.1
280 Mg 3.5 Cu	3,495	7.9	30,420	75.3	9,612[a]	26.5
SE	1,287	2.9	6,467	30.2	3,065	10.6
400 Mg 5.0 Cu	3,306	7.1	34,824	72.4	21,263	43.1
SE	1,287	2.9	6,467	30.2	3,065	10.6

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

*, Counts per minute
**, Stimulation index

(280 ug/gm). Therefore, lymphocytes from rats receiving those dietary treatments showed less proliferation than those from the other diets. At the same time, there were no statistical differences in the mean stimulation indexes among the dietary treatments.

Regression analyses were conducted using plasma magnesium or copper as the independent variable and lymphocyte stimulation response as the dependent variable. No linear or quadratic relationships existed for any of the mitogens.

Humoral Immunity

Mean plasma protein concentrations for all rats increased during the eight-week experiment. Rats consuming diets with 50 ug magnesium and 0.5 ug copper/gm or 160 ug magnesium and 2.0 ug copper/gm had significantly lower plasma protein concentrations than animals on the other dietary treatments (Table 43). Plasma protein depression caused by the 2.0 ug copper/gm diet occurred only when the diet contained 160 ug magnesium/gm.

The lower plasma protein content could be attributed to lower mean amounts of albumin, and the alpha 1 and gamma globulins. A large decrease in the gamma globulins from the severely deficient rats was observed at the eight-week sample (0.02 ± 0.03 gm/100 ml) when compared to the result of the four-week sample (0.10 ± 0.02 gm/100 ml).

Table 43. Least square mean total plasma protein, albumin and globulins (gm/100 ml) for each combined magnesium and copper dietary treatment for both sampling periods (n=10).

Diet ug/gm	Total Protein	Albumin	Globulins A1	Globulins A2	Globulins Beta	Globulins Gamma	A/G Ratio
50 Mg							
0.5 Cu	6.01[c]	3.22[c]	0.48[c]	0.52	1.73	0.06[a]	1.15
SE	0.35	0.22	0.07	0.05	0.10	0.02	0.07
160 Mg							
2.0 Cu	6.64[a]	3.76	0.85[a]	0.39	1.59	0.12	1.26
SE	0.35	0.24	0.06	0.04	0.09	0.02	0.06
280 Mg							
2.0 Cu	7.63	3.97	0.91	0.56	1.96[a]	0.14	1.18
SE	0.33	0.24	0.07	0.05	0.10	0.02	0.07
160 Mg							
3.5 Cu	7.19	4.15	1.08	0.46	1.40	0.10	1.37
SE	0.33	0.22	0.06	0.05	0.10	0.02	0.06
280 Mg							
3.5 Cu	6.96	3.93	0.98	0.45	1.47	0.11	1.31
SE	0.31	0.20	0.06	0.04	0.10	0.02	0.06
400 Mg							
5.0 Cu	7.67	4.35	1.06	0.53	1.66	0.10	1.30
SE	0.30	0.20	0.06	0.04	0.09	0.02	0.06

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

When expressed as a percent of total protein, the albumin and gamma globulin levels were similar among all dietary treatments. The percent of alpha 1 was significantly lower for the severely deficient rats with higher percentages for the alpha 2 and beta globulins. The rats consuming the 160 ug magnesium and 2.0 ug copper/gm diet had lower percentages of the alpha 2 globulins, and a slightly higher percentage of the gamma globulins, although these differences were not significant.

The severely deficient rats also had significantly lower levels of IgM and IgG as shown in Table 44. IgM was 59 percent of the control concentration and IgG was 53 percent of the control. In contrast, rats consuming moderately deficient levels of copper (2.0 ug/gm), regardless of magnesium content, had higher levels of IgM and IgG compared to the control animals. No significant changes occurred between the four- and eight-week sampling periods.

When mean comparisons were conducted on the specific antibody response data, large variation contributed to no statistical differences among the diets, although some general trends appeared. Table 45 shows that mean specific antibody titer and log titer for the 50 ug magnesium and 0.5 ug copper/gm diet were much less than that of the control. In addition, the other diet treatments generally had titers less than the control diet.

Regression analysis was used to find whether magnesium or copper status had an effect on specific antibody

Table 44. Least square mean IgM and IgG (mg/ml) for each combined magnesium and copper dietary treatment at each sampling period (n=5).

Diet ug/gm	Sampling Week 4 IgM	4 IgG	8 IgM	8 IgG	Mean SE IgM	Mean SE IgG
50 Mg 0.5 Cu	1.75	6.43	1.32	3.91	1.53[a] 0.38	5.17[a] 1.44
160 Mg 2.0 Cu	3.13	10.78	3.84	13.37	3.48 0.35	12.07 1.35
280 Mg 2.0 Cu	3.85	10.36	3.93	9.96	3.89[a] 0.39	10.16 1.30
160 Mg 3.5 Cu	2.02	7.26	2.37	11.68	2.20 0.36	9.47 1.43
280 Mg 3.5 Cu	2.36	9.35	2.61	12.06	2.49 0.35	10.71 1.29
400 Mg 5.0 Cu	2.42	7.53	2.81	12.06	2.61 0.34	9.79 1.30
Mean	2.59	8.62	2.81	10.51		
SE	0.20	0.76	0.21	0.80		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

Table 45. Least square mean specific antibody titer and log titer for each combined magnesium and copper dietary treatment at each sampling period (n=5).

Diet ug/gm	Sampling Week 4 Titer	4 Log Titer	8 Titer	8 Log Titer	Mean SE Titer	Mean SE Log Titer
50 Mg	24	8	16	5	20	6
0.5 Cu					67	3
160 Mg	73	15	110	19	92	17
2.0 Cu					60	3
280 Mg	67	13	70	14	69	13
2.0 Cu					64	3
160 Mg	297	18	14	13	156	16
3.5 Cu					64	3
280 Mg	93	20	53	12	73	16
3.5 Cu					60	3
400 Mg	113	19	143	17	128	18
5.0 Cu					60	3
Mean	111	16	68	13		
SE	35	2	36	2		

response, using plasma magnesium or copper as the independent variable and antibody titer as the dependent variable. At four weeks, no linear or quadratic relationships existed. At eight weeks, plasma magnesium and the log titer had a significant linear relationship ($P<0.01$), as well as a quadratic relationship ($P<0.05$).

Neutrophil Function

Neutrophil phagocytosis of E. coli and intracellular killing was similar for all rats, except for those consuming the 50 ug magnesium and 0.5 ug copper/gm diet. For them, significantly more neutrophils phagocytized E. coli, as presented in Table 46. Once the bacteria were ingested, killing was statistically similar, greater than 80 percent, among all dietary treatments. When a magnesium deficient medium was used, similar results were found.

Neutrophil MPO activity was significantly higher in the 50 ug magnesium and 0.5 ug copper/gm dietary treatment (Table 47). Furthermore, MPO activity highly correlated with neutrophil phagocytosis at four weeks ($r=0.64$, $P<0.001$) and at eight weeks ($r=0.62$, $P<0.001$). Neutrophil SOD was not significantly altered by the dietary treatments, as also shown in Table 47.

Summary

In general, these experiments indicated that severe single nutrient deficiencies affected immunocompetence in

Table 46. Least square mean neutrophil phagocytosis and killing (%) for each combined magnesium and copper dietary treatment for both sampling periods (n=10).

Diet ug/gm	% neutrophils without bacteria	with bacteria, not killed	with bacteria, killed
50 Mg			
0.5 Cu	20.4[c]	1.5	78.1[c]
SE	7.7	0.7	7.9
160 Mg			
2.0 Cu	52.0	3.6	44.4
SE	6.8	0.6	7.0
280 Mg			
2.0 Cu	59.0	3.4	37.6
SE	7.3	0.6	7.5
160 Mg			
3.5 Cu	62.7	2.2	35.1
SE	7.3	0.6	7.5
280 Mg			
3.5 Cu	60.9	2.3	36.7
SE	6.8	0.6	7.0
400 Mg			
5.0 Cu	68.4	2.3	30.4
SE	6.8	0.6	7.0

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

Table 47. Least square mean neutrophil myeloperoxidase (MPO) and superoxide dismutase (SOD) in U x 10^{-7}/neutrophil for each combined magnesium and copper dietary treatment at each sampling period (n=5).

Diet ug/gm	Sampling Week 4 MPO	4 SOD	8 MPO	8 SOD	Mean SE MPO	Mean SE SOD
50 Mg 0.5 Cu	1.46	222.8	2.19	206.8	1.83[c] 0.23	214.8 38.5
160 Mg 2.0 Cu	0.65	176.8	0.43	142.6	0.54 0.20	159.7 31.9
280 Mg 2.0 Cu	0.72	205.3	0.34	121.2	0.53 0.22	163.3 33.8
160 Mg 3.5 Cu	0.42	235.3	0.42	69.5	0.42 0.22	152.4 33.8
280 Mg 3.5 Cu	0.72	108.4	0.80	119.8	0.76 0.20	114.1 31.9
400 Mg 5.0 Cu	0.58	216.8	0.32	168.3	0.45 0.20	192.5 31.9
Mean	0.76	194.2	0.75	138.0		
SE	0.12	18.8	0.13	20.1		

a, different from control ($P<0.05$)
b, different from control ($P<0.01$)
c, different from control ($P<0.001$)

some way. In addition, marginal single nutrient deficiencies depressed some immunological functions, but not to the extent as severe deficiencies. The depression of immunity appeared to correlate with the degree of the nutritional deficiency, in spite of large variation that existed among the animals in each dietary treatment.

In the next chapter a summary of this research project and a discussion of each experiment will be presented.

Chapter 5

SUMMARY AND DISCUSSION

For many years it has been recognized that malnourished individuals and animals are more susceptible to disease. Dietary deficiencies generally have a negative affect on immunocompetence. Both cell-mediated and humoral immunity are depressed when the immunological mechanisms are altered, but the influence of specific nutrients remains unclear. When multiple nutrient deficiencies are present in malnutrition, it becomes difficult to define the causal relationships.

Recently, studies concerning single nutrient deficiencies were conducted to gain a clearer understanding about the involvement of nutrients in immunity. Most of these have used severely deficient animals. But few studies to date have investigated marginally deficient animals. Therefore, the objectives of this research project were to determine: 1) the effects of dietary magnesium or copper deficiencies on immune function; and 2) the effects of a combined dietary magnesium and copper deficiency on immunity. Furthermore, marginal deficiencies were examined as well as severe deficiencies.

Previous experiments with magnesium deficient animals found leukocytosis with neutrophilia and eosinophilia. In severely deficient rats thymic hyperplasia and splenomegaly

were observed, and immunoglobulin levels and antibody titers were depressed.

Other studies involving magnesium deficiencies found a depression of lymphocyte proliferation when cells were stimulated by various mitogens. DNA and protein synthesis were inhibited by magnesium deficiencies, which indicated a requirement for the nutrient for lymphocyte proliferation. Magnesium also appeared to be required for neutrophil phagocytosis. As was pointed out in the review of literature, the effect of magnesium on immunocompetence appeared to primarily be through its role in protein and DNA synthesis.

Other research reviewed suggested that the involvement of copper in the immune system may be related to its activity in metalloenzymes. Copper deficient animals had lower resistance to bacterial infections. Thymus glands were small, while spleens were enlarged in laboratory animals. Moreover, there was a reduction in antibody-producing cells, and lower responsiveness of lymphocytes to mitogen stimulation. Finally, neutrophils from copper deficient ruminants have shown impaired microbicidal activities, possibly caused by changes in SOD activity.

The studies, summarized in the review of literature, used diets depleted in magnesium or copper, to produce severe deficiencies. Marginal deficiencies, however, are becoming more clinically recognized, and the effects of these types of deficiencies on immunocompetence are unknown.

The diets in this research were designed to induce various levels of magnesium or copper deficiencies in rats, including severe, moderate and mild deficiencies. The interactions of the two marginal magnesium and copper deficiencies also were included.

Three experiments were conducted to study magnesium deficiencies, copper deficiencies, and a combined magnesium and copper deficiency. Male rats were placed on a diet with severe, moderately marginal, mildly marginal, or adequate levels of the appropriate mineral. Their nutritional status was monitored every two weeks. Plasma mineral levels, hemoglobin, hematocrit and weight gain were assessed.

After three or four weeks, half of the rats in each dietary treatment group were euthanized, while the remaining rats were euthanized at eight weeks. Immunological analyses were conducted, including T- and B-cell quantitation, lymphocyte proliferation, specific antibody response, IgG and IgM quantitation, and neutrophil phagocytosis and killing.

Statistical analyses of the nutritional data for the eight-week time period were conducted with a completely randomized split-plot design with repeated measures. Immunological data were analyzed using a factorial arrangement of diets and sampling-time periods. Pre-test data were included as covariates. In addition, regression analyses were applied to lymphocyte proliferation and antibody responses with nutritional parameters.

The remainder of this chapter will discuss the major findings of each experiment, will present the conclusions, and will offer some recommendations for future research.

Discussion

Experiment 1: Magnesium Deficiencies

The first outward indication of magnesium deficiency appeared within the first two weeks of the experiment. The characteristic clinical signs were similar to those reported by Aikawa (1981). The extensive hemorrhagic and necrotic skin lesions observed in the rats on the 50 ug magnesium/gm diet, were similar to those described by Hunt (1971) in young and mature rats after 12 days on a 66 ug magnesium/gm diet.

Nutritional Status

Early in the experiment, plasma magnesium reflected the amount of magnesium in the diet. Similar findings were reported by Hunt (1971). Later, rats consuming the marginal levels of dietary magnesium had plasma magnesium levels similar to controls.

The young growing animals had a high demand for magnesium that was utilized during protein synthesis, bone maturation, and for other physiological functions. By eight weeks, the rats had reached puberty, growth rate decreased, and the high demands for magnesium had diminished. It was

believed that for this reason, the plasma magnesium pool increased.

Although pair-fed rats consumed less food than the ad lib control rats, the level of magnesium in their diet, 400 ug/gm, was enough to support adequate magnesium status. When the total dietary magnesium intake was calculated as mg magnesium consumed/100 gm body weight, the pair-fed rats consumed levels of magnesium similar to the ad lib controls. On the other hand, the rats on the 50 ug magnesium/gm diet had consumed magnesium levels, per 100 gm body weight, that were 14 percent of the levels consumed by rats on the 400 ug magnesium/gm diets.

The severely deficient (50 ug/gm) and moderately deficient (160 ug/gm) diets also caused a decrease in bone magnesium concentrations. Hunt (1971) reported a similar effect in young rats, and found that changes in dietary magnesium in growing animals closely corresponded to changes in the concentration of bone magnesium. In this experiment, the mild deficiency (280 ug/gm) did not affect bone magnesium concentration.

The feed efficiencies explain the differences observed in weight gained among the rats receiving the dietary treatments. At four weeks, the rats receiving adequate levels of magnesium had the highest feed efficiencies, indicating that the feed was effectively utilized for growth. The feed efficiencies decreased as dietary magnesium levels decreased, thus, total growth had been lowered. At

eight weeks, the feed efficiencies for the rats on the marginal levels of magnesium improved, and total weight gains were similar to those of the ad lib control animals.

The rats on the pair-fed treatment had weight gains similar to the severely deficient rats throughout the experiment due to the caloric restriction, but their feed efficiencies were near those of the control animals. The severely deficient magnesium diet significantly affected feed efficiency and weight gain throughout the experiment. Therefore, the magnesium deficiency contributed to a less efficient utilization of the feed for growth.

The severely deficient rats had lower hemoglobin concentration and hematocrit than the rats on the other dietary treatments. It was reported that magnesium deficiency produces a hemolytic anemia, perhaps due to a loss of membrane integrity and a shortened survival time of the erythrocyte (Elin, 1980). This also might explain the lower mean hematocrit and hemoglobin concentrations found with the rats on the moderately deficient, 160 ug magnesium/gm diet.

Lymphoid Organs

Only the severely deficient, 50 ug magnesium/gm diet affected spleen weight. The significantly enlarged spleens were similar to those reported by others (Alcock, et al., 1973; Elin, 1975; McCoy and Kenney, 1975; Zieve, et al.,

1977). Thymuses were not altered, unlike those in the experiment by Alcock, et al. (1973).

Cellular Immunity

Leukocytic Effects

The typical leukocytosis, eosinophilia, and neutrophilia described in rats consuming diets with 30 to 60 ug magnesium/gm (Battifora, et al., 1968; Hass, et al., 1980) was seen in the severely deficient (50 ug/gm) rats in this experiment. The mean total WBC count for the rats on the 280 ug magnesium/gm diet also was significantly higher than those on the 160 ug magnesium/gm and 400 ug magnesium/gm diets. One rat on the 280 ug magnesium/gm diet had a high total WBC count (29.9 x 10^3/ul), which skewed the mean total WBC count. Excluding that rat, the mean total WBC count for the 280 ug magnesium/gm treatment group was 7.6 x 10^3/ul, which was similar to the control rats and those rats in the other marginally deficient group.

Lymphocyte Proliferation

Dietary magnesium deficiencies did not alter *in vitro* lymphocyte proliferation when peripheral blood mononuclear cells were stimulated by the mitogens, PHA, Con A and PWM. The incubation medium, RPMI, contained magnesium ions, which might have allowed for the normal regulation of electrolyte concentrations and normal cell proliferation. Recently, it was shown that *in vitro* alterations in extracellular Mg^{2+}

content affected cell membrane permeability and the Ca^{2+} pump, and thus affected DNA synthesis and cell proliferation (Sanui and Rubin, 1982; Abboud, et al., 1985; Okazaki, et al., 1987).

The results of this experiment support the suggested effects of extracellular magnesium on lymphocyte proliferation. The peripheral lymphocytes from the deficient rats in this experiment had response rates similar to controls. It appeared that perhaps alterations did not occur in the cell itself during magnesium deficiency. Instead, the availability of extracellular magnesium must be adequate for normal proliferation.

Gunther and Averdunk (1979) observed lower proliferation rates of magnesium deficient rat spleen and thymus cells incubated in normal RPMI. The stimulation rates in the control cells also were relatively low when compared to peripheral lymphocytes. The two authors suggested that the lower rates were due to fewer mitotically active cells present in the thymus in contrast to the peripheral lymphocytes.

Humoral Immunity

Plasma Proteins and Immunoglobulins

Total plasma protein, albumin and globulin concentrations for all rats, except the severely deficient rats, were within the normal range as reported by Mitruka and Rawnsley (1977). Rats consuming 50 ug magnesium/gm had

significantly reduced plasma proteins. This result was not surprising due to the role of magnesium in protein synthesis (Aikawa, 1981). The marginal deficiencies provided adequate amounts of magnesium for the synthesis of albumin and the globulins.

Part of the reduction in total plasma protein in the severely deficient rats was a result of a decrease in gamma globulins. IgM and IgG, which comprise most of the gamma globulins, also were decreased. Similar changes were found by other investigators (Alcock and Shils, 1974; Elin, 1975; McCoy and Kenney, 1975). In this experiment, the moderately deficient rats (160 ug/gm) also had lower IgG levels than control rats. Overall, plasma magnesium concentrations appeared to influence immunoglobulin levels, based on the correlation between the two, especially early in the experiment.

The pair-fed rats had plasma protein and immunoglobulin levels similar to the ad lib control rats. The restriction of total calories did not appear to depress protein synthesis. Therefore, the reduction in protein synthesis seen in the rats consuming 50 ug magnesium/gm was associated with the magnesium deficiency and not with a more generalized malnutrition.

Antibody Response

The rats were immunized once with a sheep red blood cell suspension, initiating a primary immune response. The

specific antibody titers appeared to correspond with IgM levels both at three weeks and at eight weeks. IgM concentrations and mean titers were lower for the severely deficient rats, but the difference in specific antibody response was not statistically significant. The tendency for the severely deficient rats to have lower titers paralleled the reduction in plasma protein synthesis.

Antibody formation is dependent on antigen presentation by the macrophage to T cells, and with the production of interleukins, stimulate B cells, which undergo transformation to blast cells (Golub, 1981). In this experiment, the percentages of peripheral T- and B-cells did not differ among the dietary treatments, nor did the proliferation rates of the peripheral blood mononuclear cells. Lower mean specific antibody titers in the severely deficient rats probably were not the result of changes in the T cell/B cell interactions, but rather were due to a failure to synthesize interleukins or the antibody protein.

Neutrophil Function

In vitro neutrophil phagocytosis of E. coli and intracellular killing was not affected by magnesium deficient diets. This was true when Mg^{2+} was adequate or deficient in the incubation medium. Extracellular magnesium has been shown to enhance neutrophil ingestion (Stossel, 1973) and NADPH oxidase activity (Johnston, et al., 1985).

The magnesium concentration in the deficient medium might have been sufficient for optimum phagocytosis.

Experiment 2: Copper Deficiencies

Three unexpected deaths occurred in the severely deficient, 0.5 ug copper/gm dietary treatment group within the first half of the experiment after obtaining blood samples. Excessive hemorrhages were found in the pericardium.

Changes in the morphology of the heart have been observed in copper deficient animals, and in rats, the heart becomes pale and friable (Fell, 1987). Studies have shown a reduction in lysyl oxidase activity, and thus, a failure of elastin and collagen biosynthesis. The strength of the cardiac tissues was reduced. Additionally, a high proportion of copper deficient rats died from rupture of the myocardium (Davis and Mertz, 1987). It was believed that for this reason, the hearts of the copper deficient rats in this experiment could not properly heal after the cardiac puncture.

Nutritional Status

Plasma copper concentrations rapidly reflected the dietary levels of copper throughout the experiment. Rats consuming 0.5 ug copper/gm were severely deficient in plasma copper throughout the eight weeks, and those consuming 2.0 ug copper/gm were moderately deficient throughout the

eight-week time period. Rats on the 3.5 ug copper/gm diet were mildly deficient in plasma copper during the first half of the experiment. Later, their plasma copper concentrations remained lower than the controls, but they were not significantly different.

Although pair-fed rats consumed less food than the ad lib control rats, the level of copper in their diet, 5.0 ug/gm, was enough to support adequate copper status. When the total dietary copper intake was calculated as mg copper consumed/100 gm body weight, the pair-fed rats consumed levels of copper similar to the ad lib controls. On the other hand, the rats on the 0.5 ug copper/gm diet had consumed copper levels, per 100 gm body weight, that were less than 10 percent of the levels consumed by rats on the 5.0 ug copper/gm diets.

A decline in Cp activity was observed, and was highly correlated with plasma copper. All levels of dietary copper, including the marginal levels, were reflected in the Cp activities. This was reported in other experiments involving copper deficient rats (Davis and Mertz, 1987; Frieden and Hsieh, 1976; Prohaska, et al., 1983).

The characteristic microcytic and hypochromic anemia due to a severe copper deficiency was present in the rats of this experiment consuming 0.5 ug copper/gm diet. Three factors known to contribute to the anemia are: 1) defects in heme synthesis caused by reduced Cp activity; 2) a delay in

the maturation of red blood cells; and 3) a shortened survival time of circulating red blood cells (Fell, 1987).

These factors may occur only in severe or prolonged copper deficiency. This may explain why the severely deficient rats on the 0.5 ug copper/gm diet were anemic throughout the experiment, while those rats on the 2.0 ug copper/gm diet only began to develop anemia after the fourth week of the experiment.

Liver copper concentration also was affected by the copper content of the diet, and was related to plasma copper and Cp activity. Others have found similar results in rats (Davis and Mertz, 1987) and in mice (Prohaska, et al., 1983).

The livers from the severely deficient rats were significantly enlarged relative to total body size. In addition, rats on the 2.0 ug copper/gm diet also tended to have larger livers in proportion to body size. Severe and prolonged marginal deficiencies in rats were shown to be accompanied by enlargement of hepatocyte mitochrondria (Fell, 1987).

The feed efficiencies may explain the differences observed in weight gain. The rats consuming marginal and adequate levels of copper had similar feed efficiencies, and total weight gains were statistically similar. The severely deficient level of copper significantly decreased feed efficiency and growth. Pair-fed rats were similar in weight

to their mates as a result of a restriction in calories, but feed utilization was better.

Lymphoid Organs

Thymus glands from the severely copper deficient rats (0.5 ug/gm), when expressed as a percent of total body weight, were significantly smaller than those from all other rats. This also was observed in mice (Prohaska, et al., 1983). In this experiment, spleen size and weight were not affected by dietary copper deficiencies.

Cellular Immunity

Leukocytic Effects

Dietary copper deficiencies did not alter total white blood cell counts, nor did they largely change the differential counts. Furthermore, T and B cell percentages were similar among all dietary treatment groups. All values were within the normal ranges for laboratory rats (Baker, et al., 1980; Carter and Bazin, 1980).

Lymphocyte Proliferation

All lymphocyte stimulation indexes (SI) for PHA, Con A, and PWM were approximately 50 percent lower for the 0.5 ug copper/gm group than for all other treatment groups, even though the differences were not statistically significant. These results were similar to those reported by Lukasewycz and Prohaska (1983) for copper deficient mice splenocytes.

The SI for PHA and PWM were lowered by the marginal deficiencies to some extent. The significant regression analysis for the PHA CPM and SI data indicated that cell proliferation, when stimulated by PHA, was dependent on the copper status of the animal, as determined by plasma copper concentrations. Lukasewycz, et al. (1987) recently suggested that the reduction of cellular proliferation appeared to be proportional to the degree of copper deficiency. This seemed to be true in regards to PHA in this experiment.

On the other hand, the mean SI for Con A in the marginally deficient diet treatments were higher than that for the control group. These differences occurred at eight weeks, when the SI for both treatments were more than twice that for the control. The differences were not statistically significant, but suggested that there might be more suppressor T cell activity in the marginally deficient copper rats.

Davis, et al. (1987) recently found that the rate of lymphoid cell proliferation after Con A stimulation was according to tissue source. Cervical lymph node cells from copper deficient rats consuming 0.6 ug copper/gm diets had higher proliferation rates, while spleen cells had lower rates of proliferation. In this experiment the peripheral lymphocytes from rats consuming 0.5 ug copper/gm diets had lower proliferation rates than those from control rats.

Humoral Immunity

Plasma Proteins and Immunoglobulins

Total plasma protein, albumin and globulin concentrations were not altered by dietary copper deficiencies. All groups were within the normal range (Mitruka and Rawnsley, 1977). Although the gamma globulin concentration for the severely deficient rats was similar to the other rats, the concentration of IgM was significantly lower in this group.

Antibody Response

Humoral-mediated immunity was found to be impaired in copper deficient mice (Prohaska and Lukasewycs, 1981; Lukasewycz and Prohaska, 1981) and rats (Koller, et al., 1987). In this study, the impairment of the primary response to heterologous RBCs was found in the marginally deficient rats after eight weeks. Regression analysis suggested that specific antibody response was dependent on the plasma copper status of the animal. Rats with low plasma copper had titers similar to rats with high plasma copper, while those with marginal levels of plasma copper had lower titers.

The difference in specific antibody response may be explained by the difference found in the marginally deficient copper diet treatments in Con A stimulation of the lymphocytes. The results indicated higher proliferation rates by Con A, suggesting greater suppressor T cell

proliferation. And, suppressor T cells act to depress antibody responses (Golub, 1981).

On the other hand, rats with low plasma copper tended to have high specific antibody titers. Rats on the severely deficient diet had a Con A SI that was approximately one-third of the control values. The reduction in suppressor T cell proliferation might be one factor which would allow for antibody responses to continue with less regulation.

Neutrophil Function

Investigations of ruminant neutrophil function have shown a depression of candidacidal activity with copper deficiencies (Jones and Suttle, 1981; Arthur, et al., 1981; Boyne and Arthur, 1981; Arthur and Boyne, 1985). In contrast, the phagocytosis and bactericidal activity of neutrophils from copper deficient rats in this experiment were not affected. Ingestion of E. coli by the neutrophils from the severely deficient rats on the 0.5 ug copper/gm diet tended to be higher, but the killing capacity was not different than that for neutrophils from the other animals.

During phagocytosis, O_2^- is produced and is involved in bacterial killing (Babior, et al., 1973). The cuproenzyme, SOD, in neutrophils scavenges the ions to protect the cell from oxidative damage. It had been proposed that impaired microbicidal activity of neutrophils from copper deficient animals was associated with a lack of SOD (Jones and Suttle, 1981). But, neutrophils from copper deficient cattle did not

have reduced SOD activity (Boyne and Arthur, 1981). In this experiment, rat neutrophil SOD activity was not altered by changes in plasma copper or Cp.

Neutrophil SOD also is required for the dismutation of O_2^- to H_2O_2. MPO reacts with H_2O_2 to form hydroxyl radicals which are potent cytotoxic agents against microorganisms (Klebanoff, et al., 1984). It was postulated that perhaps a defect in MPO, a heme enzyme, may result from decreased Cp ferroxidase activity from copper deficiency. It was observed that MPO activity significantly correlated with neutrophil microbicidal activity. But, a reduction of Cp activity in the copper deficient rats did not affect neutrophil MPO activity.

Experiment 3: Magnesium and Copper Deficiencies

Rats on the severely deficient diet with 50 ug magnesium and 0.5 ug copper/gm began to show clinical signs of the magnesium deficiency within the first two weeks. These were similar to those described in Experiment 1.

During the fifth week of the experiment, seizure activity was observed in one of the severely deficient rats. A study of seizure susceptibility with magnesium deficiency found that rats fed diets with 60 ug magnesium/gm and adequate levels of calcium and phosphorus were highly prone to seizures (Chaistitwanich, el al., 1987). The authors found that all rats consuming the diet had low serum magnesium and elevated levels of serum potassium. This

condition favored seizures which were observed in all rats in the treatment group.

Nutritional Status

Both plasma magnesium and copper concentrations reflected the amount of mineral in the diet. The differences were apparent at two weeks, and continued throughout the eight-week time period. Cp activity followed plasma copper concentrations. Additionally, bone magnesium and liver copper content were indicative of dietary magnesium and copper content, respectively. These patterns were similar to those in the first two experiments.

The combined severe magnesium and copper deficiency produced anemia that by eight weeks was more severe than the anemia found in rats deficient in only one of the minerals. The anemia appeared to be hemolytic, microcytic and hypochromic. Reticulocytosis was common. Contributing to the severe anemic state was diminished cell membrane integrity from the magnesium deficiency (Elin, 1980), and a defect in heme synthesis from the copper deficiency (Fell, 1987). Both mineral deficiencies delayed maturation and shortened survival time of the erythrocyte (Elin, 1980; Fell, 1987). Marginal deficiencies did not produce anemia.

The combined magnesium and copper marginal deficiencies did not affect growth of the rats. Only those consuming the severely deficient diet with 50 ug magnesium and 0.5 ug copper/gm gained significantly less weight. The feed

efficiency for those rats was lower, indicating that utilization of the feed was poor.

Lymphoid Organs

In this experiment, both the spleen and thymus of the severely deficient rats were affected. Enlarged spleens, common in magnesium deficiencies (Alcock, et al., 1973; Elin, 1975; McCoy and Kenney, 1975; Zieve, et al., 1977) and also reported in copper deficiencies (Prohaska, et al., 1983) were found in the rats on the 50 ug magnesium and 0.5 ug copper/gm diet. Thymus glands were small, as reported in copper deficiencies (Prohaska, et al., 1983), rather than enlarged as reported in magnesium deficiencies (Alcock, et al., 1973). The other treatments did not affect spleen and thymus weights.

Cellular Immunity

Leukocytic Effects

Leukocytosis and neutrophilia was present in the rats on the 50 ug magnesium and 0.5 ug copper/gm diet during the first half of the experiment. This corresponded to the effects reported with a magnesium deficiency (Battifora, et al., 1968; Hass et al., 1980). Although the nutritional status of the rats remained poor at eight weeks, the total WBC and neutrophil counts improved to come within the normal range. McCreary, et al., (1967) found a tendency of elevated leukocyte counts to diminish after two weeks. They found

that to sustain leukocytosis in rats, the magnesium content of the diet had to be reduced to 30 ug/gm.

A combined severe deficiency of magnesium (50 ug/gm) and copper (0.5 ug/gm), and a combined moderate deficiency of magnesium (160 ug/gm) and copper (2.0 ug/gm) elevated T cell percentages. This did not happen when either mineral was deficient in Experiments 1 and 2. The elevated T cell percentage has not been reported in the major literature for either mineral. All other dietary deficiency treatments were similar to the control treatment.

Lymphocyte Proliferation

Proliferation responses of cells stimulated with PHA tended to be lower in the 50 ug magnesium and 0.5 ug copper/gm diet treatment group. Lower rates have been reported by others with splenocytes and thymocytes from magnesium deficient rats (Gunther and Averdunk, 1979), and splenocytes from copper deficient mice (Lukasewycz and Prohaska, 1983).

The stimulation indexes for Con A were higher in the 50 ug magnesium and 0.5 ug copper/gm treatment and in the 280 ug magnesium and 2.0 ug copper/gm treatment. In Experiment 2, marginal copper deficiencies also increased Con A stimulation rates, but the severe copper deficiency decreased the SI. The differences, however, were not statistically significant.

Significantly lower proliferation rates were found when the lymphocytes from rats consuming diets deficient in magnesium (50 ug/gm and 280 ug/gm) and in copper (0.5 ug/gm and 2.0 ug/gm) were stimulated with PWM. Splenocytes from mice consuming a 0.6 ug copper/gm diet, also had reduced proliferation rates when stimulated with PWM (Lukasewycz and Prohaska, 1983).

Together, the higher Con A and lower PWM rates would suggest that cell-mediated responses may be altered in the treatment groups with the severe combined deficiencies and the marginal deficiencies with 2.0 ug copper/gm.

Humoral Immunity

Plasma Proteins and Immunoglobulins

Total plasma proteins were reduced by a combined severe deficiency of magnesium (50 ug/gm) and copper (0.5 ug/gm), and by a combined moderate deficiency of magnesium (160 ug/gm) and copper (2.0 ug/gm). The effect of the severe deficiency was expected because of the role of magnesium in protein synthesis (Aikawa, 1981). In Experiment 1, the marginal magnesium deficiencies did not affect total plasma proteins. Yet, in combination with the copper deficiency, the 160 ug/gm level of magnesium did reduce total plasma proteins.

The gamma globulins were dramatically reduced during the eight-week experiment in the 50 ug magnesium and 0.5 ug copper/gm treatment. This was consistent with the

significantly lower concentrations of IgM and IgG. Similar decreases were found in Experiment 1 with the 50 ug magnesium/gm treatment, and in Experiment 2 for IgM in the 0.5 ug copper/gm treatment.

Antibody Response

Mean specific antibody response for the treatments appeared to follow the lymphocyte stimulation rates with PWM. Those treatments with low transformation in response to PWM also tended to have low specific antibody titers. PWM is a stimulator of B cells, resulting in B cell proliferation. Therefore, reduced B cell proliferation and differentiation would decrease antibody production (Golub, 1981).

A primary antibody response was measured, rather than a secondary response because the rats were immunized once, six days prior to sampling. The decrease in IgM in the 50 ug magnesium and 0.5 ug copper/gm treatment corresponded with the low specific antibody titer. This was not true, however, for the rats on the diets with 2.0 ug copper/gm, that had higher IgM levels.

Neutrophil Function

A greater percentage of neutrophils from the severely deficient rats consuming the 50 ug magnesium and 0.5 ug copper/gm diet ingested E. coli than neutrophils from rats in all other treatment groups. But, bactericidal activity did not differ among the treatments. When the neutrophils

were incubated in a magnesium deficient medium, the results were the same.

In Experiment 2, slightly more neutrophils from rats consuming a diet with 0.5 ug copper/gm ingested the E.coli, when compared to neutrophils from the other treatments, but the difference was not statistically significant. In this experiment, with the additional magnesium deficiency, the ingestion by neutrophils was significantly higher.

Neutrophils produce a "respiratory burst" when stimulated, altering oxygen metabolism to generate microbicidal oxidants. The membrane bound enzyme that catalyzes the reduction of oxygen to O_2^- is NADPH oxidase (Cross, et al., 1985; Markert, et al., 1985).

Oxidase activity is low in resting cells, and the phagocyte must be stimulated to transform the latent activity to an expressed activity (Forman and Thomas, 1986). The transformation and activation processes must occur for greater phagocytic responses. These appear to be receptor-mediated processes, and involve depolarization of membrane potential. Changes in Ca^{2+} also appear to occur in the stimulation process. The specific factors involved are uncertain.

In this experiment, alterations in Mg^{2+} and Cu^{2+}, with possible changes in Ca^{2+} or Zn^{2+} metabolism might have affected the oxidase system and phagocytosis.

In this experiment, as in the second experiment, neutrophil SOD activity was not altered by copper

deficiencies. On the other hand, MPO activity was significantly higher in the neutrophils from rats on the 50 ug magnesium and 0.5 ug copper/gm diet. Furthermore, MPO activity significantly correlated with neutrophil microbicidal activity.

It was thought, however, that in the deficient treatment groups, there would have been a reduction of MPO activity because of depressed Cp ferroxidase activity from the copper deficiency. Cp did not affect another heme enzyme, catalase, at four weeks. But later, Cp and catalase activity were correlated. Effects from the ferroxidase activity of Cp may occur after a prolonged deficiency.

Conclusions

The dietary treatments used in these experiments were successful in inducing severe and marginal deficiencies in the rats as indicated by the nutritional parameters. Differences among all treatments were found midway through the experiment. By the end of the experiments, rats consuming severely deficient diets (50 ug magnesium/gm or 0.5 ug copper/gm) remained in a poor nutritional state. Rats consuming the marginally deficient diets tended to improve, with rats on the mildly deficient diets (280 ug magnesium/gm or 3.5 ug copper/gm) becoming similar in nutritional status to the control animals.

The severe dietary deficiencies of magnesium and/or copper affected immunocompetence of the rats in these

experiments. In general, lower specific antibody titers and depressed lymphocyte proliferation responses were found. However, many of the results were not statistically significant because of the large variation in the responses. A magnesium deficiency appeared to have its effect on protein synthesis, while a copper deficiency altered cellular proliferation by some unknown mechanism. Furthermore, a severe copper deficiency increased neutrophil phagocytosis of bacteria, but killing was not changed.

Marginal deficiencies did not always alter immune function. One finding, even though not statistically significant, was the increased response of the lymphocytes to Con A stimulation with a corresponding decrease in antibody production. Overall, other cellular and humoral immune responses, and neutrophil functions were not affected by marginal deficiencies.

In the final analysis, the results suggested that within some physiological interval, a marginal deficiency of magnesium or copper would not effect immune function. But, beyond that interval a deficiency would become critical to optimum immunocompetence.

Recommendations for Further Research

One limitation of the experiments in this research project were the number of rats used in each treatment group. The large variation in the immunological responses contributed to not being able to detect significant

differences. This can only be overcome by larger sample sizes. The resources also must be present, however, to handle the larger number of animals and samples.

Unlike previous studies, this project investigated the effects of marginal single nutrient deficiencies on immune function. More experiments are required to explore these effects of not only magnesium and copper, but also other essential nutrients. Furthermore, the effects of long-term marginal deficiencies is unknown. Other experiments may study the deficiencies in older animals and for a time period that extends beyond eight weeks.

It appeared that marginal copper deficiencies might increase T suppressor cell proliferation and differentiation. Confirmation of these results is indicated. Another study could determine the mechanism that might be altered by the change in copper status could be done. Furthermore, more information is required about how single nutrients are involved in immune mechanisms.

One experiment in this research project investigated a combined magnesium and copper deficiency. Ingestion by neutrophils from rats deficient in both nutrients was greater than those from control animals. The biochemical reactions responsible for this change may be observed in another study. Other combinations of minerals could be explored to provide more information on nutrient interactions in the immune system.

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APPENDIX

Table 48. Summary of the statistical model for plasma magnesium in Experiment 1, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	4	25.92	0.0001
Rat(Diet)	35	4.33	
Week	5	10.82	0.0001
Diet*Week	20	5.69	0.0001
Error	92	5.50	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	0.001	-			
280	0.001	0.01	-		
400, ad lib	0.001	0.001	NS	-	
400, pair fed	0.001	0.001	NS	NS	-
WEEK Initial	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	NS	NS	NS	-	
400, pair fed	NS	NS	NS	NS	-
WEEK 2	PR > T				
50	-				
160	0.001	-			
280	0.001	0.01	-		
400, ad lib	0.001	0.001	NS	-	
400, pair fed	0.001	0.001	NS	NS	-

Table 48. (continued)

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
WEEK 3	PR > T				
50	-				
160	0.001	-			
280	0.001	NS	-		
400, ad lib	0.001	0.01	NS	-	
400, pair fed	0.001	0.01	NS	NS	-
WEEK 4	PR > T				
50	-				
160	0.001	-			
280	0.001	0.01	-		
400, ad lib	0.001	0.01	NS	-	
400, pair fed	0.001	0.05	NS	NS	-
WEEK 6	PR > T				
50	-				
160	0.01	-			
280	0.001	NS	-		
400, ad lib	0.001	0.01	NS	-	
400, pair fed	0.001	0.001	NS	NS	-
WEEK 8	PR > T				
50	-				
160	0.001	-			
280	0.001	NS	-		
400, ad lib	0.001	NS	NS	-	
400, pair fed	0.001	NS	NS	NS	-

Table 49. Summary of the statistical model for hemoglobin in Experiment 1, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	4	30.79	0.0004
Rat(Diet)	35	39.64	
Week	5	254.82	0.0001
Diet*Week	20	40.55	0.0162
Error	92	94.83	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	0.01	-			
280	0.001	NS	-		
400, ad lib	0.001	NS	NS	-	
400, pair fed	0.001	NS	NS	NS	-
WEEK Initial	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	NS	NS	NS	-	
400, pair fed	NS	NS	NS	NS	-
WEEK 2	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	NS	NS	NS	-	
400, pair fed	0.01	NS	NS	NS	-

Table 49. (continued)

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
WEEK 3	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	NS	NS	NS	-	
400, pair fed	NS	NS	NS	NS	-
WEEK 4	PR > T				
50	-				
160	0.01	-			
280	0.001	NS	-		
400, ad lib	0.01	NS	NS	-	
400, pair fed	0.01	NS	NS	NS	-
WEEK 6	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	0.001	0.001	0.01	-	
400, pair fed	0.05	NS	NS	0.01	-
WEEK 8	PR > T				
50	-				
160	NS	-			
280	0.01	NS	-		
400, ad lib	0.001	NS	NS	-	
400, pair fed	0.01	NS	NS	NS	-

Table 50. Summary of the statistical model for hematocrit in Experiment 1, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	4	177.60	0.0006
Rat(Diet)	35	246.23	
Week	5	782.68	0.0001
Diet*Week	20	431.31	0.0001
Error	92	584.35	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	NS	-			
280	0.001	0.01	-		
400, ad lib	0.001	0.01	NS	-	
400, pair fed	0.01	0.05	NS	NS	-
WEEK Initial	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	NS	NS	NS	-	
400, pair fed	NS	NS	NS	NS	-
WEEK 2	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	NS	NS	NS	-	
400, pair fed	NS	NS	NS	NS	-

Table 50. (continued)

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
WEEK 3	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	NS	0.05	NS	-	
400, pair fed	NS	0.05	NS	NS	-
WEEK 4	PR > T				
50	-				
160	NS	-			
280	0.001	0.05	-		
400, ad lib	NS	NS	0.05	-	
400, pair fed	0.01	NS	NS	NS	-
WEEK 6	PR > T				
50	-				
160	0.001	-			
280	NS	0.001	-		
400, ad lib	0.001	0.001	0.01	-	
400, pair fed	0.05	0.001	NS	0.05	-
WEEK 8	PR > T				
50	-				
160	NS	-			
280	0.01	NS	-		
400, ad lib	0.01	NS	NS	-	
400, pair fed	0.05	NS	NS	NS	-

Table 51. Summary of the statistical model for weight gain in Experiment 1, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	13147.25	0.0021
Week	1	63833.68	0.0001
Diet*Week	4	3421.98	0.2520
Error	29	17459.42	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	0.05	-			
280	0.01	NS	-		
400, ad lib	0.001	NS	NS	-	
400, pair fed	NS	NS	0.05	0.01	-

Table 52. Summary of the statistical model for feed efficiency in Experiment 1, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	0.01	0.0401
Week	1	0.03	0.0001
Diet*Week	4	0.01	0.0772
Error	29	0.04	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	0.01	0.05	NS	-	
400, pair fed	NS	NS	NS	NS	-

Table 53. Summary of the statistical model for bone magnesium in Experiment 1, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	4	32.45	0.0001
Week	1	0.62	0.0013
Diet*Week	4	1.20	0.0011
Error	29	1.42	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	0.001	-			
280	0.001	0.01	-		
400, ad lib	0.001	0.001	0.05	-	
400, pair fed	0.001	0.001	NS	NS	-
WEEK 3	PR > T				
50	-				
160	0.001	-			
280	0.001	NS	-		
400, ad lib	0.001	0.01	0.05	-	
400, pair fed	0.001	0.01	NS	NS	-
WEEK 8	PR > T				
50	-				
160	0.001	-			
280	0.001	0.001	-		
400, ad lib	0.001	0.001	NS	-	
400, pair fed	0.001	0.001	NS	NS	-

Table 54. Summary of the statistical model for spleen weight in Experiment 1, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	0.06	0.0001
Week	1	0.01	0.0034
Diet*Week	4	0.01	0.1870
Error	29	0.02	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	0.001	-			
280	0.001	NS	-		
400, ad lib	0.001	NS	NS	-	
400, pair fed	0.001	NS	NS	NS	-

Table 55. Summary of the statistical model for thymus weight in Experiment 1.

Source	DF	SS	PR > F
Diet	4	0.002	0.8710
Week	1	0.045	0.0001
Diet*Week	4	0.004	0.5877
Error	29	0.044	

Table 56. Summary of the statistical model for total white blood cell count in Experiment 1, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	196.92	0.0372
Week	1	55.66	0.0782
Diet*Week	4	64.57	0.4399
Covariate	1	43.27	0.1182
Error	28	466.40	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	0.05	-			
280	NS	NS	-		
400, ad lib	0.05	NS	NS	-	
400, pair fed	0.01	NS	NS	NS	-

Table 57. Summary of the statistical model for eosinophils in Experiment 1, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	4	70.60	0.0028
Week	1	84.04	0.0001
Diet*Week	4	77.76	0.0016
Covariate	1	0.00	0.9784
Error	28	94.25	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	NS	-			
280	0.001	NS	-		
400, ad lib	0.01	NS	NS	-	
400, pair fed	0.001	NS	NS	NS	-
WEEK 3	PR > T				
50	-				
160	0.05	-			
280	0.001	0.05	-		
400, ad lib	0.01	0.001	NS	-	
400, pair fed	0.001	0.01	NS	NS	-
WEEK 8	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	NS	NS	NS	-	
400, pair fed	NS	NS	NS	NS	-

Table 58. Summary of the statistical model for lymphocytes in Experiment 1, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	826.97	0.0360
Week	1	12.34	0.6763
Diet*Week	4	641.65	0.0821
Covariate	1	2.10	0.8631
Error	28	1941.07	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	0.05	NS	0.05	-	
400, pair fed	0.05	NS	0.01	NS	-

Table 59. Summary of the statistical models for basophils, neutrophils and monocytes in Experiment 1.

basophils:

Source	DF	SS	PR > F
Diet	4	0.48	0.5940
Week	1	0.03	0.6953
Diet*Week	4	1.04	0.2176
Covariate	1	0.03	0.6587
Error	28	4.72	

neutrophils:

Source	DF	SS	PR > F
Diet	4	534.36	0.0755
Week	1	83.87	0.2316
Diet*Week	4	575.62	0.0601
Covariate	1	31.67	0.4587
Error	28	1570.58	

monocytes:

Source	DF	SS	PR > F
Diet	4	6.85	0.4635
Week	1	0.00	0.9848
Diet*Week	4	2.84	0.8183
Covariate	1	0.06	0.8595
Error	28	51.86	

Table 60. Summary of the statistical models for T cells and B cells in Experiment 1.

T cells:

Source	DF	SS	PR > F
Block: Day	3	226.75	0.4317
Diet	4	105.48	0.8546
Week	1	18.21	0.6367
Diet*Week	4	58.90	0.9442
Error	26	2071.92	

B cells:

Source	DF	SS	PR > F
Block: Day	3	81.92	0.7013
Diet	4	217.33	0.4520
Week	1	146.95	0.1213
Diet*Week	4	101.57	0.7762
Error	26	1489.50	

Table 61. Summary of the statistical models for PHA counts per minute and the stimulation index in Experiment 1.

CPM:

Source	DF	SS	PR > F
Block: Day	3	12488365	0.9800
Diet	4	70846967	0.9019
Week	1	96714	0.9703
Diet*Week	4	272156575	0.4291
Error	26	1782223447	

SI:

Source	DF	SS	PR > F
Block: Day	3	41.74	0.9880
Diet	4	191.64	0.9627
Week	1	56.66	0.6801
Diet*Week	4	862.38	0.6242
Error	26	8471.32	

Table 62. Summary of the statistical models for Con A counts per minute and the stimulation index in Experiment 1.

CPM:

Source	DF	SS	PR > F
Block: Day	3	24715086354	0.0278
Diet	4	1502673843	0.9555
Week	1	7111655964	0.0913
Diet*Week	4	3844102775	0.7960
Error	26	60155288862	

SI:

Source	DF	SS	PR > F
Block: Day	3	81832	0.0280
Diet	4	33336	0.3839
Week	1	26847	0.0727
Diet*Week	4	19377	0.6446
Error	26	199512	

Table 63. Summary of the statistical models for PWM counts per minute and the stimulation index in Experiment 1.

CPM:

Source	DF	SS	PR > F
Block: Day	3	943268211	0.1307
Diet	4	241499696	0.8105
Week	1	1526717974	0.0040
Diet*Week	4	310308310	0.7308
Error	26	3976791084	

SI:

Source	DF	SS	PR > F
Block: Day	3	4464	0.0422
Diet	4	2225	0.3449
Week	1	6601	0.0009
Diet*Week	4	1428	0.5646
Error	26	12310	

Table 64. Summary of the statistical model for total plasma proteins in Experiment 1, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	16.40	0.0148
Week	1	33.12	0.0001
Diet*Week	4	9.45	0.1007
Covariate	1	1.66	0.2288
Error	28	30.74	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	0.001	-			
280	NS	0.05	-		
400, ad lib	0.05	NS	NS	-	
400, pair fed	0.05	NS	NS	NS	-

Table 65. Summary of the statistical model for albumin in Experiment 1, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	4	4.93	0.0142
Week	1	9.27	0.0001
Diet*Week	4	4.83	0.0155
Covariate	1	0.10	0.5773
Error	28	9.15	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	0.01	-			
280	0.01	NS	-		
400, ad lib	0.05	NS	NS	-	
400, pair fed	0.01	NS	NS	NS	-
WEEK 3	PR > T				
50	-				
160	NS	-			
280	NS	NS	-		
400, ad lib	NS	NS	NS	-	
400, pair fed	0.01	0.05	NS	0.05	-
WEEK 8	PR > T				
50	-				
160	0.001	-			
280	0.01	NS	-		
400, ad lib	0.05	NS	NS	-	
400, pair fed	NS	0.01	NS	NS	-

Table 66. Summary of the statistical model for alpha 1 globulin in Experiment 1, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	2.11	0.0012
Week	1	0.56	0.0176
Diet*Week	4	0.49	0.2565
Covariate	1	0.06	0.4242
Error	28	2.45	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	0.001	-			
280	NS	0.01	-		
400, ad lib	0.01	NS	NS	-	
400, pair fed	NS	0.01	NS	NS	-

Table 67. Summary of the statistical models for the alpha 2 and beta globulins in Experiment 1.

alpha 2:

Source	DF	SS	PR > F
Diet	4	0.11	0.3699
Week	1	0.20	0.0093
Diet*Week	4	0.04	0.8131
Covariate	1	0.00	0.7738
Error	28	0.72	

beta:

Source	DF	SS	PR > F
Diet	4	0.65	0.2790
Week	1	1.70	0.0008
Diet*Week	4	0.35	0.5776
Covariate	1	0.09	0.3861
Error	28	3.36	

Table 68. Summary of the statistical model for gamma globulin in Experiment 1, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	0.08	0.0330
Week	1	0.06	0.0054
Diet*Week	4	0.04	0.2405
Covariate	1	0.01	0.3003
Error	28	0.18	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	NS	-			
280	0.01	NS	-		
400, ad lib	0.05	NS	NS	-	
400, pair fed	0.01	NS	NS	NS	-

Table 69. Summary of the statistical model for the albumin/globulin ratio in Experiment 1, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	0.34	0.0151
Week	1	0.01	0.5229
Diet*Week	4	0.15	0.1892
Covariate	1	0.07	0.0859
Error	28	0.64	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	NS	-			
280	0.05	NS	-		
400, ad lib	NS	NS	NS	-	
400, pair fed	0.01	NS	NS	0.01	-

Table 70. Summary of the statistical model for IgM in Experiment 1, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	1.39	0.0416
Week	1	0.13	0.3039
Diet*Week	4	0.36	0.5670
Covariate	1	0.41	0.0754
Error	28	3.40	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	0.05	-			
280	0.01	NS	-		
400, ad lib	0.01	NS	NS	-	
400, pair fed	0.01	NS	NS	NS	-

Table 71. Summary of the statistical model for IgG in Experiment 1, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	277.36	0.0039
Week	1	58.24	0.0515
Diet*Week	4	65.26	0.3499
Covariate	1	249.92	0.0002
Error	28	394.10	

Mg Diet (ug/gm)	50	160	280	400 ad lib	400 pair fed
MAIN EFFECT	PR > T				
50	-				
160	NS	-			
280	0.01	NS	-		
400, ad lib	0.01	0.05	NS	-	
400, pair fed	0.001	0.01	NS	NS	-

Table 72. Summary of the statistical models for antibody titer and log titer in Experiment 1.

antibody titer:

Source	DF	SS	PR > F
Diet	4	70932.45	0.3459
Week	1	22481.57	0.2338
Diet*Week	4	36628.33	0.6634
Covariate	1	8260.84	0.4668
Error	28	424993.83	

log titer:

Source	DF	SS	PR > F
Diet	4	461.67	0.2591
Week	1	186.54	0.1435
Diet*Week	4	52.62	0.9569
Covariate	1	0.09	0.9734
Error	28	2305.91	

Table 73. Summary of the statistical models for neutrophil phagocytosis and killing in Experiment 1.

without bacteria:

Source	DF	SS	PR > F
Block: Day	3	498.90	0.1089
Diet	4	216.00	0.5841
Week	1	1.26	0.8978
Diet*Week	4	365.41	0.3252
Error	26	1941.89	

with bacteria, not killed:

Source	DF	SS	PR > F
Block: Day	3	8.80	0.3204
Diet	4	4.78	0.7368
Week	1	0.01	0.9384
Diet*Week	4	4.04	0.7915
Error	26	62.24	

with bacteria, killed:

Source	DF	SS	PR > F
Block: Day	3	504.31	0.0963
Diet	4	213.33	0.5712
Week	1	0.85	0.9143
Diet*Week	4	375.36	0.2931
Error	26	1865.55	

Table 74. Summary of the statistical model for plasma copper in Experiment 2, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	4	8.40	0.0001
Rat(Diet)	45	4.75	
Week	3	3.37	0.0001
Diet*Week	12	6.16	0.0001
Error	105	5.42	

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
MAIN EFFECT	PR > T				
0.5	-				
2.0	0.01	-			
3.5	0.001	0.01	-		
5.0, ad lib	0.001	0.001	NS	-	
5.0, pair fed	0.001	0.001	NS	NS	-
WEEK Initial	PR > T				
0.5	-				
2.0	NS	-			
3.5	NS	NS	-		
5.0, ad lib	NS	NS	NS	-	
5.0, pair fed	NS	NS	NS	NS	-
WEEK 2	PR > T				
0.5	-				
2.0	0.01	-			
3.5	0.001	0.01	-		
5.0, ad lib	0.001	0.001	NS	-	
5.0, pair fed	0.001	0.001	0.05	NS	-

Table 74. (continued)

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
WEEK 4	PR > T				
0.5	-				
2.0	0.001	-			
3.5	0.001	0.001	-		
5.0, ad lib	0.001	0.001	0.05	-	
5.0, pair fed	0.001	0.001	NS	NS	-
WEEK 8	PR > T				
0.5	-				
2.0	0.05	-			
3.5	0.001	0.01	-		
5.0, ad lib	0.001	0.001	NS	-	
5.0, pair fed	0.001	0.001	NS	NS	-

Table 75. Summary of the statistical model for ceruloplasmin in Experiment 2, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	4	10275.47	0.0001
Rat(Diet)	45	4865.33	
Week	3	2042.51	0.0003
Diet*Week	12	5825.29	0.0001
Error	80	7635.38	

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
MAIN EFFECT	PR > T				
0.5	-				
2.0	NS	-			
3.5	0.001	0.001	-		
5.0, ad lib	0.001	0.001	0.05	-	
5.0, pair fed	0.001	0.001	0.01	NS	-
WEEK Initial	PR > T				
0.5	-				
2.0	NS	-			
3.5	NS	NS	-		
5.0, ad lib	NS	NS	NS	-	
5.0, pair fed	NS	NS	NS	NS	-
WEEK 2	PR > T				
0.5	-				
2.0	NS	-			
3.5	0.001	0.01	-		
5.0, ad lib	0.001	0.001	0.05	-	
5.0, pair fed	0.001	0.001	0.001	NS	-

Table 75. (continued)

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
WEEK 4	PR > T				
0.5	-				
2.0	NS	-			
3.5	NS	NS	-		
5.0, ad lib	0.001	0.001	0.01	-	
5.0, pair fed	0.01	0.05	NS	NS	-
WEEK 8	PR > T				
0.5	-				
2.0	NS	-			
3.5	0.01	0.001	-		
5.0, ad lib	0.01	0.001	NS	-	
5.0, pair fed	0.001	0.001	NS	NS	-

Table 76. Summary of the statistical model for catalase in Experiment 2.

Source	DF	SS	PR > F
Diet	4	967.39	0.7112
Rat(Diet)	44	19918.49	
Week	1	232.95	0.4433
Diet*Week	4	1922.19	0.3217
Error	15	5634.69	

Table 77. Summary of the statistical model for hemoglobin in Experiment 2, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	4	114.30	0.0001
Rat(Diet)	45	78.17	
Week	3	152.69	0.0001
Diet*Week	12	66.25	0.0001
Error	105	145.20	

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
MAIN EFFECT	PR > T				
0.5	-				
2.0	0.001	-			
3.5	0.001	NS	-		
5.0, ad lib	0.001	NS	NS	-	
5.0, pair fed	0.001	0.01	0.01	NS	-
WEEK Initial	PR > T				
0.5	-				
2.0	NS	-			
3.5	NS	NS	-		
5.0, ad lib	NS	NS	NS	-	
5.0, pair fed	NS	NS	NS	NS	-
WEEK 2	PR > T				
0.5	-				
2.0	0.01	-			
3.5	0.001	NS	-		
5.0, ad lib	0.001	NS	NS	-	
5.0, pair fed	0.001	0.001	0.01	0.01	-

Table 77. (continued)

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
WEEK 4	PR > T				
0.5	-				
2.0	0.001	-			
3.5	0.001	NS	-		
5.0, ad lib	0.001	NS	NS	-	
5.0, pair fed	0.001	NS	NS	NS	-
WEEK 8	PR > T				
0.5	-				
2.0	0.05	-			
3.5	NS	NS	-		
5.0, ad lib	0.01	NS	0.05	-	
5.0, pair fed	0.001	NS	0.01	NS	-

Table 78. Summary of the statistical model for hematocrit in Experiment 2, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	4	669.76	0.0001
Rat(Diet)	45	414.96	
Week	3	596.87	0.0001
Diet*Week	12	464.90	0.0001
Error	105	745.93	

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
MAIN EFFECT	PR > T				
0.5	-				
2.0	0.01	-			
3.5	0.001	0.05	-		
5.0, ad lib	0.001	0.001	NS	-	
5.0, pair fed	0.001	0.001	0.01	NS	-
WEEK Initial	PR > T				
0.5	-				
2.0	NS	-			
3.5	NS	NS	-		
5.0, ad lib	NS	NS	NS	-	
5.0, pair fed	NS	NS	NS	NS	-
WEEK 2	PR > T				
0.5	-				
2.0	0.001	-			
3.5	0.001	NS	-		
5.0, ad lib	0.001	NS	NS	-	
5.0, pair fed	0.001	0.01	NS	0.05	-

Table 78. (continued)

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
WEEK 4	PR > T				
0.5	-				
2.0	0.001	-			
3.5	0.001	0.01	-		
5.0, ad lib	0.001	0.001	NS	-	
5.0, pair fed	0.001	0.001	NS	NS	-
WEEK 8	PR > T				
0.5	-				
2.0	NS	-			
3.5	NS	NS	-		
5.0, ad lib	0.01	0.01	0.05	-	
5.0, pair fed	0.001	0.01	0.05	NS	-

Table 79. Summary of the statistical model for weight gain in Experiment 2, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	4	27773.38	0.0001
Week	1	63785.67	0.0001
Diet*Week	4	8846.18	0.0098
Error	36	20384.41	

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
MAIN EFFECT	PR > T				
0.5	-				
2.0	0.01	-			
3.5	0.001	0.05	-		
5.0, ad lib	0.001	NS	NS	-	
5.0, pair fed	NS	0.01	0.001	0.001	-
WEEK 4	PR > T				
0.5	-				
2.0	NS	-			
3.5	NS	NS	-		
5.0, ad lib	NS	NS	NS	-	
5.0, pair fed	NS	NS	NS	NS	-
WEEK 8	PR > T				
0.5	-				
2.0	0.001	-			
3.5	0.001	0.05	-		
5.0, ad lib	0.001	NS	NS	-	
5.0, pair fed	NS	0.001	0.001	0.001	-

Table 80. Summary of the statistical model for feed efficiency in Experiment 2, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	0.01	0.0205
Week	1	0.10	0.0001
Diet*Week	4	0.00	0.6314
Error	36	0.02	

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
MAIN EFFECT	PR > T				
0.5	-				
2.0	NS	-			
3.5	0.01	NS	-		
5.0, ad lib	0.05	NS	NS	-	
5.0, pair fed	NS	NS	0.01	NS	-

Table 81. Summary of the statistical model for liver weight in Experiment 2, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	4.50	0.0261
Week	1	0.89	0.0276
Diet*Week	4	0.67	0.7601
Error	36	12.93	

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
MAIN EFFECT	PR > T				
0.5	-				
2.0	0.05	-			
3.5	0.01	NS	-		
5.0, ad lib	0.01	NS	NS	-	
5.0, pair fed	0.01	NS	NS	NS	-

Table 82. Summary of the statistical model for liver copper in Experiment 2, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	43.23	0.0001
Week	1	0.30	0.3039
Diet*Week	4	0.31	0.8901
Error	36	10.00	

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
MAIN EFFECT	PR > T				
0.5	-				
2.0	0.001	-			
3.5	0.001	0.001	-		
5.0, ad lib	0.001	0.001	NS	-	
5.0, pair fed	0.001	0.01	NS	0.05	-

Table 83. Summary of the statistical model for spleen weight in Experiment 2.

Source	DF	SS	PR > F
Diet	4	0.002	0.6993
Week	1	0.006	0.0179
Diet*Week	4	0.004	0.4108
Error	36	0.036	

Table 84. Summary of the statistical model for thymus weight in Experiment 2, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	0.03	0.0059
Week	1	0.03	0.0002
Diet*Week	4	0.00	0.8430
Error	36	0.06	

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
MAIN EFFECT	PR > T				
0.5	-				
2.0	0.01	-			
3.5	0.001	NS	-		
5.0, ad lib	0.01	NS	NS	-	
5.0, pair fed	0.05	NS	NS	NS	-

Table 85. Summary of the statistical models for the total white blood cell count and the differential counts in Experiment 2.

total white blood cells:

Source	DF	SS	PR > F
Diet	4	4.63	0.9233
Week	1	0.18	0.8526
Diet*Week	4	4.69	0.9219
Covariate	1	0.07	0.9088
Error	34	176.12	

basophils:

Source	DF	SS	PR > F
Diet	4	1.30	0.1167
Week	1	0.79	0.0346
Diet*Week	4	1.59	0.0647
Covariate	1	0.08	0.4946
Error	34	5.52	

eosinophils:

Source	DF	SS	PR > F
Diet	4	0.26	0.9952
Week	1	8.10	0.0175
Diet*Week	4	0.14	0.9984
Covariate	1	0.36	0.6033
Error	34	44.14	

Table 85. (continued)

neutrophils:

Source	DF	SS	PR > F
Diet	4	90.66	0.5933
Week	1	72.84	0.1412
Diet*Week	4	53.41	0.7959
Covariate	1	3.36	0.7484
Error	34	1091.34	

lymphocytes:

Source	DF	SS	PR > F
Diet	4	74.55	0.7407
Week	1	233.63	0.0180
Diet*Week	4	87.16	0.6815
Covariate	1	4.48	0.7327
Error	34	1284.87	

monocytes:

Source	DF	SS	PR > F
Diet	4	0.58	0.9925
Week	1	13.20	0.0227
Diet*Week	4	10.25	0.3697
Covariate	1	0.49	0.6472
Error	34	78.81	

Table 86. Summary of the statistical models for T cells and B cells in Experiment 2.

T cells:

Source	DF	SS	PR > F
Block: Day	4	288.41	0.5899
Diet	4	465.93	0.3515
Week	1	44.49	0.5123
Diet*Week	4	445.80	0.3737
Error	31	3138.69	

B cells:

Source	DF	SS	PR > F
Block: Day	4	62.43	0.8754
Diet	4	102.08	0.7421
Week	1	46.02	0.3539
Diet*Week	4	34.06	0.9551
Error	31	1610.42	

Table 87. Summary of the statistical models for PHA counts per minute and the stimulation index in Experiment 2.

CPM:

Source	DF	SS	PR > F
Block: Day	4	22970048	0.5122
Diet	4	50924465	0.1433
Week	1	13017250	0.1782
Diet*Week	4	17851279	0.6308
Error	31	212661910	

SI:

Source	DF	SS	PR > F
Block: Day	4	228.09	0.6113
Diet	4	449.68	0.2773
Week	1	278.36	0.0782
Diet*Week	4	175.33	0.7199
Error	31	2600.91	

Table 88. Summary of the statistical models for Con A counts per minute and the stimulation index in Experiment 2.

CPM:

Source	DF	SS	PR > F
Block: Day	4	1158925921	0.0921
Diet	4	705233024	0.2778
Week	1	1626364233	0.0014
Diet*Week	4	772753304	0.2361
Error	31	4082847406	

SI:

Source	DF	SS	PR > F
Block: Day	4	32568.54	0.0812
Diet	4	15827.95	0.3664
Week	1	46758.76	0.0010
Diet*Week	4	18154.71	0.2987
Error	31	109844.01	

Table 89. Summary of the statistical models for PWM counts per minute and the stimulation index in Experiment 2.

CPM:

Source	DF	SS	PR > F
Block: Day	4	66957893	0.0101
Diet	4	27140030	0.1950
Week	1	13606216	0.0815
Diet*Week	4	6715423	0.8071
Error	31	130110498	

SI:

Source	DF	SS	PR > F
Block: Day	4	1082.86	0.0017
Diet	4	201.98	0.4041
Week	1	502.79	0.0031
Diet*Week	4	130.17	0.6190
Error	31	1510.09	

Table 90. Summary of the statistical models for the total plasma proteins, albumin and the globulins in Experiment 2.

total protein:

Source	DF	SS	PR > F
Diet	4	15.49	0.6050
Week	1	20.96	0.0619
Diet*Week	4	30.10	0.2760
Covariate	1	2.69	0.4942
Error	34	191.24	

albumin:

Source	DF	SS	PR > F
Diet	4	2.07	0.9056
Week	1	6.42	0.0850
Diet*Week	4	7.97	0.4334
Covariate	1	1.47	0.4015
Error	34	69.41	

alpha 1:

Source	DF	SS	PR > F
Diet	4	0.71	0.4238
Week	1	0.03	0.7029
Diet*Week	4	1.72	0.0688
Covariate	1	0.02	0.7241
Error	34	6.08	

Table 90. (continued)

alpha 2:

Source	DF	SS	PR > F
Diet	4	0.26	0.4169
Week	1	0.14	0.1420
Diet*Week	4	0.26	0.4001
Covariate	1	0.00	0.8226
Error	34	2.16	

beta:

Source	DF	SS	PR > F
Diet	4	2.10	0.2788
Week	1	2.00	0.0307
Diet*Week	4	0.79	0.7331
Covariate	1	0.16	0.5342
Error	34	13.40	

gamma:

Source	DF	SS	PR > F
Diet	4	0.02	0.7385
Week	1	0.12	0.0036
Diet*Week	4	0.07	0.2793
Covariate	1	0.03	0.1222
Error	34	0.42	

Table 91. Summary of the statistical model for IgM in Experiment 2, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	4	5.12	0.0012
Week	1	1.31	0.0205
Diet*Week	4	0.65	0.5788
Covariate	1	1.85	0.0068
Error	34	7.55	

Cu Diet (ug/gm)	0.5	2.0	3.5	5.0 ad lib	5.0 pair fed
MAIN EFFECT	PR > T				
0.5	-				
2.0	0.01	-			
3.5	0.001	NS	-		
5.0, ad lib	0.001	NS	NS	-	
5.0, pair fed	0.001	NS	NS	NS	-

Table 92. Summary of the statistical model for IgG in Experiment 2.

Source	DF	SS	PR > F
Diet	4	184.74	0.0756
Week	1	145.90	0.0104
Diet*Week	4	50.89	0.6361
Covariate	1	19.89	0.3234
Error	34	673.50	

Table 93. Summary of the statistical models for antibody titer and log titer in Experiment 2.

antibody titer:

Source	DF	SS	PR > F
Diet	4	1282.27	0.3580
Week	1	8.40	0.8643
Diet*Week	4	756.96	0.6186
Covariate	1	2110.24	0.0100
Error	34	9631.16	

log titer:

Source	DF	SS	PR > F
Diet	4	134.43	0.2193
Week	1	1.71	0.7828
Diet*Week	4	155.89	0.1601
Covariate	1	176.68	0.0079
Error	34	753.47	0.7828

Table 94. Summary of the statistical models for neutrophil phagocytosis and killing in Experiment 2.

without bacteria:

Source	DF	SS	PR > F
Block: Day	4	3101.38	0.0105
Diet	4	1397.21	0.1580
Week	1	3.73	0.8913
Diet*Week	4	913.63	0.3456
Error	31	6082.48	

with bacteria, not killed:

Source	DF	SS	PR > F
Block: Day	4	235.18	0.2331
Diet	4	110.28	0.6028
Week	1	2685.06	0.0001
Diet*Week	4	105.97	0.6208
Error	31	1234.18	

with bacteria, killed:

Source	DF	SS	PR > F
Block: Day	4	4412.46	0.0014
Diet	4	1456.16	0.1371
Week	1	2938.85	0.0005
Diet*Week	4	1090.33	0.2521
Error	31	5970.00	

Table 95. Summary of the statistical models for myeloperoxidase (MPO) and superoxide dismutase (SOD) in Experiment 2.

MPO:

Source	DF	SS	PR > F
Diet	4	1.09	0.6733
Week	1	8.25	0.0002
Diet*Week	4	1.55	0.5093
Error	35	16.19	

SOD:

Source	DF	SS	PR > F
Diet	4	23136.90	0.2963
Week	1	5.30	0.9729
Diet*Week	4	12541.57	0.6011
Error	35	158112.93	

Table 96. Summary of the statistical model for plasma magnesium in Experiment 3, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	12.85	0.0001
Rat(Diet)	54	1.50	
Week	3	10.53	0.0001
Diet*Week	15	9.43	0.0001
Error	98	3.04	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	0.001	-			
160 Mg 3.5 Cu	0.001	NS	0.01	-		
280 Mg 3.5 Cu	0.001	0.001	NS	0.001	-	
400 Mg 5.0 Cu	0.001	0.001	NS	0.001	NS	-
WEEK Initial	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.05	-				
280 Mg 2.0 Cu	NS	0.001	-			
160 Mg 3.5 Cu	0.05	0.001	NS	-		
280 Mg 3.5 Cu	NS	0.001	NS	NS	-	
400 Mg 5.0 Cu	NS	0.001	NS	NS	NS	-
WEEK 2	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	0.001	-			
160 Mg 3.5 Cu	0.001	0.05	0.05	-		
280 Mg 3.5 Cu	0.001	0.001	NS	0.01	-	
400 Mg 5.0 Cu	0.001	0.001	NS	0.01	NS	-

Table 96. (continued)

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	0.001	-			
160 Mg 3.5 Cu	0.001	0.01	NS	-		
280 Mg 3.5 Cu	0.001	0.001	NS	NS	-	
400 Mg 5.0 Cu	0.001	0.01	NS	NS	NS	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	0.01	-			
160 Mg 3.5 Cu	0.001	NS	0.05	-		
280 Mg 3.5 Cu	0.001	0.001	NS	0.01	-	
400 Mg 5.0 Cu	0.001	0.001	NS	0.01	NS	-

Table 97. Summary of the statistical model for bone magnesium in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	47.98	0.0001
Week	1	0.11	0.4751
Diet*Week	5	2.00	0.1179
Error	44	9.37	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	0.001	-			
160 Mg 3.5 Cu	0.001	NS	0.001	-		
280 Mg 3.5 Cu	0.001	0.01	NS	0.05	-	
400 Mg 5.0 Cu	0.001	0.001	NS	0.01	NS	-

Table 98. Summary of the statistical model for plasma copper in Experiment 3, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	12.82	0.0001
Rat(Diet)	54	3.48	
Week	3	8.69	0.0001
Diet*Week	15	5.77	0.0001
Error	98	3.81	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.01	NS	-			
160 Mg 3.5 Cu	0.001	0.001	0.001	-		
280 Mg 3.5 Cu	0.001	0.001	0.001	NS	-	
400 Mg 5.0 Cu	0.001	0.001	0.001	0.01	0.001	-
WEEK Initial	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	NS	NS	NS	NS	-	
400 Mg 5.0 Cu	NS	NS	NS	NS	NS	-
WEEK 2	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	0.001	0.001	0.001	-		
280 Mg 3.5 Cu	0.001	0.001	0.001	NS	-	
400 Mg 5.0 Cu	0.001	0.001	0.001	0.01	0.01	-

Table 98. (continued)

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	0.05	NS	-			
160 Mg 3.5 Cu	0.001	0.001	0.001	-		
280 Mg 3.5 Cu	0.001	0.001	0.001	NS	-	
400 Mg 5.0 Cu	0.001	0.001	0.001	0.05	0.01	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.01	NS	-			
160 Mg 3.5 Cu	0.001	0.001	0.001	-		
280 Mg 3.5 Cu	0.001	0.001	0.001	NS	-	
400 Mg 5.0 Cu	0.001	0.001	0.001	NS	0.05	-

Table 99. Summary of the statistical model for plasma ceruloplasmin in Experiment 3, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	26439.12	0.0001
Rat(Diet)	54	7885.34	
Week	3	4341.09	0.0001
Diet*Week	15	8194.71	0.0001
Error	98	9368.70	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	0.001	0.001	0.001	-		
280 Mg 3.5 Cu	0.001	0.001	0.001	NS	-	
400 Mg 5.0 Cu	0.001	0.001	0.001	0.01	NS	-
WEEK Initial	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.01	NS	NS	-		
280 Mg 3.5 Cu	NS	0.001	0.001	0.01	-	
400 Mg 5.0 Cu	NS	0.001	0.001	0.001	NS	-
WEEK 2	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	0.001	0.001	0.001	-		
280 Mg 3.5 Cu	0.001	0.001	0.001	NS	-	
400 Mg 5.0 Cu	0.001	0.001	0.001	NS	0.05	-

Table 99. (continued)

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	0.01	0.05	0.05	-		
280 Mg 3.5 Cu	0.001	0.001	0.001	0.05	-	
400 Mg 5.0 Cu	0.001	0.001	0.001	0.01	NS	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.05	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	0.001	0.001	0.001	-		
280 Mg 3.5 Cu	0.001	0.001	0.001	NS	-	
400 Mg 5.0 Cu	0.001	0.001	0.001	NS	NS	-

Table 100. Summary of the statistical model for liver weight in Experiment 3, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	14.62	0.0001
Week	1	0.40	0.0148
Diet*Week	5	5.48	0.0010
Error	44	9.54	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	0.05	NS	NS	NS	-	
400 Mg 5.0 Cu	0.01	NS	NS	NS	NS	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-

Table 101. Summary of the statistical model for liver copper in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	58.85	0.0001
Week	1	0.65	0.1640
Diet*Week	5	0.96	0.7070
Error	44	14.34	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	0.01	0.001	-		
280 Mg 3.5 Cu	0.001	0.001	0.001	NS	-	
400 Mg 5.0 Cu	0.001	0.001	0.001	0.01	0.05	-

Table 102. Summary of the statistical model for hemoglobin in Experiment 3, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	136.63	0.0001
Rat(Diet)	54	163.44	
Week	3	73.94	0.0001
Diet*Week	15	86.78	0.0039
Error	98	228.42	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-
WEEK Initial	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	NS	NS	NS	NS	-	
400 Mg 5.0 Cu	NS	NS	NS	NS	NS	-
WEEK 2	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	0.05	NS	NS	NS	-	
400 Mg 5.0 Cu	0.05	NS	NS	NS	NS	-

Table 102. (continued)

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.05	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	0.05	NS	NS	NS	-	
400 Mg 5.0 Cu	NS	NS	NS	NS	NS	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-

Table 103. Summary of the statistical model for hematocrit in Experiment 3, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	795.98	0.0001
Rat(Diet)	54	814.44	
Week	3	933.67	0.0001
Diet*Week	15	756.44	0.0001
Error	98	1254.36	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-
WEEK Initial	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	NS	NS	NS	NS	-	
400 Mg 5.0 Cu	NS	NS	NS	NS	NS	-
WEEK 2	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.05	-				
280 Mg 2.0 Cu	0.05	NS	-			
160 Mg 3.5 Cu	0.05	NS	NS	-		
280 Mg 3.5 Cu	0.05	NS	NS	NS	-	
400 Mg 5.0 Cu	NS	NS	NS	NS	NS	-

Table 103. (continued)

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.01	-				
280 Mg 2.0 Cu	0.05	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	0.05	NS	NS	NS	-	
400 Mg 5.0 Cu	0.01	NS	NS	NS	NS	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-

Table 104. Summary of the statistical model for weight gain in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	45666.66	0.0001
Week	1	105342.30	0.0001
Diet*Week	5	3516.25	0.5908
Error	44	41280.31	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-

Table 105. Summary of the statistical model for feed efficiency in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	0.04	0.0001
Week	1	0.01	0.0001
Diet*Week	5	0.00	0.8712
Error	44	0.04	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-

Table 106. Summary of the statistical model for spleen weight in Experiment 3, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	0.14	0.0001
Week	1	0.03	0.0001
Diet*Week	5	0.02	0.0326
Error	44	0.05	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	0.05	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-

Table 107. Summary of the statistical model for thymus weight in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	0.02	0.0086
Week	1	0.10	0.0001
Diet*Week	5	0.00	0.9874
Error	44	0.04	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	0.01	NS	NS	-		
280 Mg 3.5 Cu	0.01	NS	NS	NS	-	
400 Mg 5.0 Cu	0.01	NS	NS	NS	NS	-

Table 108. Summary of the statistical model for total white blood cell count in Experiment 3, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	92.53	0.0031
Week	1	0.03	0.9394
Diet*Week	5	110.95	0.0009
Covariate	1	79.96	0.0001
Error	43	186.44	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	0.05	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.01	NS	NS	NS	NS	-
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	NS	NS	NS	NS	-	
400 Mg 5.0 Cu	NS	NS	NS	NS	NS	-

Table 109. Summary of the statistical model for basophils in Experiment 3, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	9.27	0.0260
Week	1	12.13	0.0001
Diet*Week	5	9.38	0.0247
Covariate	1	2.61	0.0515
Error	43	27.94	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	0.01	-		
280 Mg 3.5 Cu	NS	NS	NS	NS	-	
400 Mg 5.0 Cu	0.05	0.05	0.01	NS	0.05	-
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	NS	NS	NS	NS	-	
400 Mg 5.0 Cu	NS	NS	NS	NS	NS	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	0.05	0.05	0.01	-		
280 Mg 3.5 Cu	NS	NS	NS	0.05	-	
400 Mg 5.0 Cu	0.001	0.001	0.001	NS	0.01	-

Table 110. Summary of the statistical model for eosinophils in Experiment 3, and the least square mean probabilities for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	20.85	0.0745
Week	1	4.41	0.1367
Diet*Week	5	28.45	0.0218
Covariate	1	0.88	0.5012
Error	43	82.42	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	NS	NS	NS	NS	-	
400 Mg 5.0 Cu	NS	NS	NS	NS	NS	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.01	-				
280 Mg 2.0 Cu	0.01	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.05	NS	NS	NS	NS	-

Table 111. Summary of the statistical model for neutrophils in Experiment 3, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	1180.84	0.0002
Week	1	28.11	0.3877
Diet*Week	5	474.52	0.0403
Covariate	1	3.41	0.7627
Error	43	1587.19	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	NS	NS	NS	NS	-	
400 Mg 5.0 Cu	NS	NS	NS	NS	NS	-

Table 112. Summary of the statistical model for lymphocytes in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	1461.52	0.0007
Week	1	16.39	0.5892
Diet*Week	5	334.24	0.3220
Covariate	1	0.23	0.9492
Error	43	2380.77	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.01	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-

Table 113. Summary of the statistical model for monocytes in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	28.45	0.0548
Week	1	12.06	0.0301
Diet*Week	5	4.73	0.8501
Covariate	1	7.06	0.0934
Error	43	103.09	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.01	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	0.05	NS	NS	-		
280 Mg 3.5 Cu	NS	0.05	NS	NS	-	
400 Mg 5.0 Cu	NS	0.05	NS	NS	NS	-

Table 114. Summary of the statistical model for T cells in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Block: Day	4	859.82	0.0850
Diet	5	1469.55	0.0209
Week	1	2079.55	0.0001
Diet*Week	5	507.44	0.4055
Error	40	3888.38	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	0.01	NS	-			
160 Mg 3.5 Cu	0.05	NS	NS	-		
280 Mg 3.5 Cu	0.01	NS	NS	NS	-	
400 Mg 5.0 Cu	0.01	0.05	NS	NS	NS	-

Table 115. Summary of the statistical model for B cells in Experiment 3.

Source	DF	SS	PR > F
Block: Day	4	1155.19	0.0074
Diet	5	619.49	0.1470
Week	1	574.54	0.0070
Diet*Week	5	412.34	0.3454
Error	40	2842.76	

Table 116. Summary of the statistical models for PHA counts per minute and the stimulation index in Experiment 3.

CPM:

Source	DF	SS	PR > F
Block: Day	4	87712680	0.0696
Diet	5	29392437	0.6246
Error	17	140896022	

SI:

Source	DF	SS	PR > F
Block: Day	4	872.66	0.0068
Diet	5	75.58	0.8721
Error	17	724.43	

Table 117. Summary of the statistical models for Con A counts per minute and the stimulation index in Experiment 3.

CPM:

Source	DF	SS	PR > F
Block: Day	4	6208931567	0.0012
Diet	5	771874498	0.6052
Error	17	3554979024	

SI:

Source	DF	SS	PR > F
Block: Day	4	78489.92	0.0137
Diet	5	4851.88	0.9521
Error	17	77349.75	

Table 118. Summary of the statistical model for PWM counts per minute in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Block: Day	4	1133994972	0.0033
Diet	5	922945778	0.0150
Error	17	798449757	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	0.05	0.05	0.05	-		
280 Mg 3.5 Cu	NS	NS	NS	0.05	-	
400 Mg 5.0 Cu	0.01	0.01	0.01	NS	0.05	-

Table 119. Summary of the statistical model for the PWM stimulation index in Experiment 3.

Source	DF	SS	PR > F
Block: Day	4	15291.67	0.0019
Diet	5	2346.69	0.5431
Error	17	9561.50	

Table 120. Summary of the statistical model for total plasma proteins in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	14.55	0.0158
Week	1	3.96	0.0436
Diet*Week	5	1.08	0.9447
Covariate	1	1.07	0.2853
Error	43	39.40	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	0.01	NS	-			
160 Mg 3.5 Cu	0.05	NS	NS	-		
280 Mg 3.5 Cu	0.05	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	0.05	NS	NS	NS	-

Table 121. Summary of the statistical model for the albumin/globulin ratio in Experiment 3.

Source	DF	SS	PR > F
Diet	5	0.26	0.2263
Week	1	0.05	0.2479
Diet*Week	5	0.23	0.2938
Covariate	1	0.13	0.0597
Error	43	1.54	

Table 122. Summary of the statistical model for albumin in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	6.13	0.0178
Week	1	2.16	0.0242
Diet*Week	5	0.28	0.9814
Covariate	1	0.06	0.6943
Error	43	17.02	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	0.05	NS	-			
160 Mg 3.5 Cu	0.01	NS	NS	-		
280 Mg 3.5 Cu	0.05	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-

Table 123. Summary of the statistical model for alpha 1 globulin in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	1.96	0.0001
Week	1	0.09	0.1162
Diet*Week	5	0.09	0.7732
Covariate	1	0.11	0.0792
Error	43	1.50	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	0.01	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	0.05	NS	NS	NS	-

Table 124. Summary of the statistical model for alpha 2 globulin in Experiment 3.

Source	DF	SS	PR > F
Diet	5	0.18	0.0832
Week	1	0.04	0.1365
Diet*Week	5	0.07	0.5535
Covariate	1	0.15	0.0044
Error	43	0.72	

Table 125. Summary of the statistical model for beta globulin in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	1.80	0.0039
Week	1	0.33	0.0570
Diet*Week	5	0.23	0.7601
Covariate	1	0.03	0.5466
Error	43	3.77	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	0.01	-			
160 Mg 3.5 Cu	0.05	NS	0.001	-		
280 Mg 3.5 Cu	NS	NS	0.001	NS	-	
400 Mg 5.0 Cu	NS	NS	0.05	NS	NS	-

Table 126. Summary of the statistical model for gamma globulin in Experiment 3, and the least square mean probabilities for the diet main effect and for the diet*week interaction.

Source	DF	SS	PR > F
Diet	5	0.03	0.0312
Week	1	0.00	0.9325
Diet*Week	5	0.03	0.0368
Covariate	1	0.04	0.0001
Error	43	0.10	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.01	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	0.05	NS	NS	NS	-	
400 Mg 5.0 Cu	NS	NS	NS	NS	NS	-
WEEK 4	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	NS	-				
280 Mg 2.0 Cu	NS	NS	-			
160 Mg 3.5 Cu	NS	NS	NS	-		
280 Mg 3.5 Cu	NS	NS	NS	NS	-	
400 Mg 5.0 Cu	NS	NS	NS	NS	NS	-
WEEK 8	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	NS	0.05	0.01	-		
280 Mg 3.5 Cu	0.01	NS	NS	NS	-	
400 Mg 5.0 Cu	0.01	NS	NS	NS	NS	-

Table 127. Summary of the statistical model for IgM in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	26.97	0.0015
Week	1	0.71	0.4325
Diet*Week	5	1.61	0.9195
Covariate	1	20.70	0.0001
Error	43	48.82	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	NS	0.05	0.01	-		
280 Mg 3.5 Cu	NS	NS	0.05	NS	-	
400 Mg 5.0 Cu	0.05	NS	0.05	NS	NS	-

Table 128. Summary of the statistical model for IgG in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	230.28	0.0300
Week	1	48.53	0.0953
Diet*Week	5	84.62	0.4208
Covariate	1	83.70	0.0303
Error	43	717.12	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.05	NS	-			
160 Mg 3.5 Cu	0.05	NS	NS	-		
280 Mg 3.5 Cu	0.01	NS	NS	NS	-	
400 Mg 5.0 Cu	0.05	NS	NS	NS	NS	-

Table 129. Summary of the statistical models for antibody titer and log titer in Experiment 3.

antibody titer:

Source	DF	SS	PR > F
Diet	5	97392.87	0.7349
Week	1	26130.45	0.3937
Diet*Week	5	151250.35	0.5161
Covariate	1	32429.45	0.3425
Error	43	1513723.15	

log titer:

Source	DF	SS	PR > F
Diet	5	777.51	0.1422
Week	1	80.85	0.3445
Diet*Week	5	213.06	0.7880
Covariate	1	157.01	0.1899
Error	43	3804.89	

Table 130. Summary of the statistical model for neutrophil phagocytosis in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Block: Day	4	1921.59	0.4035
Diet	5	12309.25	0.0008
Week	1	2574.66	0.0238
Diet*Week	5	1455.23	0.6821
Error	40	18648.81	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.01	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-

Table 131. Summary of the statistical model for neutrophil killing in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Block: Day	4	1933.66	0.4312
Diet	5	12519.93	0.0011
Week	1	2579.68	0.0278
Diet*Week	5	1655.06	0.6494
Error	40	19801.62	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.01	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-

Table 132. Summary of the statistical model for myeloperoxidase in Experiment 3, and the least square mean probabilities for the diet main effect.

Source	DF	SS	PR > F
Diet	5	11.99	0.0004
Week	1	0.00	0.9479
Diet*Week	5	0.69	0.5493
Error	44	18.40	

Diet (ug/gm)	50 Mg 0.5 Cu	160 Mg 2.0 Cu	280 Mg 2.0 Cu	160 Mg 3.5 Cu	280 Mg 3.5 Cu	400 Mg 5.0 Cu
MAIN EFFECT	PR > T					
50 Mg 0.5 Cu	-					
160 Mg 2.0 Cu	0.001	-				
280 Mg 2.0 Cu	0.001	NS	-			
160 Mg 3.5 Cu	0.001	NS	NS	-		
280 Mg 3.5 Cu	0.001	NS	NS	NS	-	
400 Mg 5.0 Cu	0.001	NS	NS	NS	NS	-

Table 133. Summary of the statistical model for superoxide dismutase in Experiment 3.

Source	DF	SS	PR > F
Diet	5	52115.75	0.4150
Week	1	42386.42	0.0474
Diet*Week	5	43959.01	0.5126
Error	43	437253.26	

Table 134. Correlation coefficients (r) and probabilities (P) for plasma magnesium, spleen and thymus weights, and white blood cell count (WBC) in Experiment 1 at three weeks and eight weeks.

three weeks:

		plasma Mg	spleen wt	thymus wt	WBC
plasma Mg	r=	-			
	P=				
spleen wt	r=	-0.61	-		
	P=	0.01			
thymus wt	r=	-0.02	0.08	-	
	P=	NS	NS		
WBC	r=	-0.69	0.35	0.14	-
	P=	0.001	NS	NS	

eight weeks:

		plasma Mg	spleen wt	thymus wt	WBC
plasma Mg	r=	-			
	P=				
spleen wt	r=	-0.78	-		
	P=	0.001			
thymus wt	r=	0.08	-0.15	-	
	P=	NS	NS		
WBC	r=	-0.30	0.32	-0.17	-
	P=	NS	NS	NS	

Table 135. Correlation coefficients (r) and probabilities (P) for plasma magnesium, IgM, IgG, and antibody titer in Experiment 1 at three weeks and eight weeks.

three weeks:

		plasma Mg	IgM	IgG	antibody titer
plasma Mg	r=	-			
	P=				
IgM	r=	0.63	-		
	P=	0.01			
IgG	r=	0.58	0.25	-	
	P=	0.01	NS		
antibody titer	r=	0.29	0.49	0.06	-
	P=	NS	0.05	NS	

eight weeks:

		plasma Mg	IgM	IgG	antibody titer
plasma Mg	r=	-			
	P=				
IgM	r=	0.57	-		
	P=	0.01			
IgG	r=	0.04	0.34	-	
	P=	NS	NS		
antibody titer	r=	0.15	0.45	0.23	-
	P=	NS	0.05	NS	

Table 136. Correlation coefficients (r) and probabilities (P) for plasma copper, spleen and thymus weights, and white blood cell count (WBC) in Experiment 2 at four weeks and eight weeks.

four weeks:

		plasma Cu	spleen wt	thymus wt	WBC
plasma Cu	r=	-			
	P=				
spleen wt	r=	0.13	-		
	P=	NS			
thymus wt	r=	0.35	0.46	-	
	P=	NS	0.05		
WBC	r=	0.27	0.39	0.47	-
	P=	NS	NS	0.05	

eight weeks:

		plasma Cu	spleen wt	thymus wt	WBC
plasma Cu	r=	-			
	P=				
spleen wt	r=	-0.18	-		
	P=	NS			
thymus wt	r=	0.20	-0.24	-	
	P=	NS	NS		
WBC	r=	-0.17	-0.46	0.27	-
	P=	NS	0.05	NS	

Table 137. Correlation coefficients (r) and probabilities (P) for plasma copper, IgM, IgG, and antibody titer in Experiment 2 at four weeks and eight weeks.

four weeks:

		plasma Cu	IgM	IgG	antibody titer
plasma Cu	r=	-			
	P=				
IgM	r=	0.50	-		
	P=	0.01			
IgG	r=	-0.23	-0.10	-	
	P=	NS	NS		
antibody titer	r=	-0.001	-0.09	-0.27	-
	P=	NS	NS	NS	

eight weeks:

		plasma Cu	IgM	IgG	antibody titer
plasma Cu	r=	-			
	P=				
IgM	r=	0.32	-		
	P=	NS			
IgG	r=	-0.46	0.19	-	
	P=	0.05	NS		
antibody titer	r=	-0.16	-0.42	-0.29	-
	P=	NS	NS	NS	

Table 138. Correlation coefficients (r) and probabilities (P) for plasma copper, ceruloplasmin (Cp), neutrophil killing, superoxide dismutase (SOD), and myeloperoxidase (MPO) in Experiment 2 at four weeks and eight weeks.

four weeks:

		plasma Cu	Cp	neutrophil killing	SOD	MPO
plasma Cu	r=	-				
	P=					
Cp	r=	0.71	-			
	P=	0.001				
neutrophil killing	r=	-0.17	-0.24	-		
	P=	NS	NS			
SOD	r=	0.36	0.24	-0.11	-	
	P=	NS	NS	NS		
MPO	r=	0.09	0.11	0.52	-0.04	-
	P=	NS	NS	0.01	NS	

eight weeks:

		plasma Cu	Cp	neutrophil killing	SOD	MPO
plasma Cu	r=	-				
	P=					
Cp	r=	0.64	-			
	P=	0.01				
neutrophil killing	r=	-0.14	-0.30	-		
	P=	NS	NS			
SOD	r=	0.17	0.05	0.44	-	
	P=	NS	NS	0.05		
MPO	r=	0.07	0.04	0.40	0.002	-
	P=	NS	NS	NS	NS	

Table 139. Correlation coefficients (r) and probabilities (P) for plasma copper, ceruloplasmin (Cp), liver weight and copper concentration, and catalase in Experiment 2 at four weeks and eight weeks.

four weeks:

		plasma Cu	Cp	liver wt	liver Cu	catalase
plasma Cu	r=	-				
	P=					
Cp	r=	0.71	-			
	P=	0.001				
liver wt	r=	-0.43	-0.11	-		
	P=	0.05	NS			
liver Cu	r=	0.76	0.66	-0.55	-	
	P=	0.001	0.001	0.01		
catalase	r=	-0.18	-0.33	-0.49	-0.11	-
	P=	NS	NS	0.01	NS	

eight weeks:

		plasma Cu	Cp	liver wt	liver Cu	catalase
plasma Cu	r=	-				
	P=					
Cp	r=	0.64	-			
	P=	0.01				
liver wt	r=	-0.74	-0.40	-		
	P=	0.001	NS			
liver Cu	r=	0.87	0.62	-0.72	-	
	P=	0.001	0.01	0.001		
catalase	r=	0.27	0.30	-0.01	0.31	-
	P=	NS	NS	NS	NS	

Table 140. Correlation coefficients (r) and probabilities (P) for plasma magnesium and copper, spleen and thymus weights, and total white blood cell counts (WBC) in Experiment 3 at four weeks and eight weeks.

four weeks:

		plasma Mg	plasma Cu	spleen wt	thymus wt	WBC
plasma Mg	r=	-				
	P=					
plasma Cu	r=	0.65	-			
	P=	0.001				
spleen wt	r=	-0.73	-0.45	-		
	P=	0.001	0.01			
thymus wt	r=	0.32	0.18	-0.29	-	
	P=	NS	NS	NS		
WBC	r=	-0.66	-0.57	0.75	0.08	-
	P=	0.001	0.001	0.001	NS	

eight weeks:

		plasma Mg	plasma Cu	spleen wt	thymus wt	WBC
plasma Mg	r=	-				
	P=					
plasma Cu	r=	0.63	-			
	P=	0.001				
spleen wt	r=	-0.59	-0.35	-		
	P=	0.001	NS			
thymus wt	r=	0.37	0.34	-0.17	-	
	P=	NS	NS	NS		
WBC	r=	0.05	-0.11	0.01	0.50	-
	P=	NS	NS	NS	0.01	

Table 141. Correlation coefficients (r) and probabilities (P) for plasma magnesium and copper, IgM, IgG, and antibody titer in Experiment 3 at four weeks and eight weeks.

four weeks:

		plasma Mg	plasma Cu	IgM	IgG	antibody titer
plasma Mg	r=	-				
	P=					
plasma Cu	r=	0.65	-			
	P=	0.001				
IgM	r=	0.15	-0.11	-		
	P=	NS	NS			
IgG	r=	0.18	-0.15	0.63	-	
	P=	NS	NS	0.001		
antibody	r=	0.32	0.43	-0.01	-0.03	-
titer	P=	NS	0.05	NS	NS	

eight weeks:

		plasma Mg	plasma Cu	IgM	IgG	antibody titer
plasma Mg	r=	-				
	P=					
plasma Cu	r=	0.63	-			
	P=	0.001				
IgM	r=	0.25	-0.07	-		
	P=	NS	NS			
IgG	r=	0.47	0.41	0.46	-	
	P=	0.01	0.05	0.05		
antibody	r=	0.22	0.26	0.39	0.18	-
titer	P=	NS	NS	0.05	NS	

Table 142. Correlation coefficients (r) and probabilities (P) for plasma magnesium and copper, ceruloplasmin (Cp), neutrophil killing, superoxide dismutase (SOD), and myeloperoxidase (MPO) in Experiment 3 at four weeks and eight weeks.

four weeks:

		plasma Mg	plasma Cu	Cp	neutrophil killing	SOD	MPO
plasma Cu	r=	0.65	-				
	P=	0.001					
Cp	r=	0.50	0.86	-			
	P=	0.01	0.001				
neutrophil killing	r=	-0.36	-0.25	-0.36	-		
	P=	NS	NS	NS			
SOD	r=	0.12	0.06	-0.10	0.18	-	
	P=	NS	NS	NS	NS		
MPO	r=	-0.29	-0.16	-0.13	0.64	0.09	-
	P=	NS	NS	NS	0.001	NS	

eight weeks:

		plasma Mg	plasma Cu	Cp	neutrophil killing	SOD	MPO
plasma Cu	r=	0.63	-				
	P=	0.001					
Cp	r=	0.60	0.94	-			
	P=	0.001	0.001				
neutrophil killing	r=	-0.55	-0.46	-0.43	-		
	P=	0.01	0.01	0.05			
SOD	r=	-0.09	-0.17	-0.11	0.11	-	
	P=	NS	NS	NS	NS		
MPO	r=	-0.60	-0.41	-0.35	0.62	0.36	-
	P=	0.001	0.05	NS	0.001	NS	

Table 143. Correlation coefficients (r) and probabilities (P) for plasma magnesium and copper, ceruloplasmin (CP), liver weight and copper concentration, and catalase (Cat) in Experiment 3 at four weeks and eight weeks.

four weeks:

		plasma Mg	plasma Cu	Cp	liver wt	liver Cu	Cat
plasma Cu	r=	0.65	-				
	P=	0.001					
Cp	r=	0.50	0.86	-			
	P=	0.01	0.001				
liver wt	r=	-0.61	-0.55	-0.42	-		
	P=	0.001	0.01	0.05			
liver Cu	r=	0.78	0.93	0.80	-0.60	-	
	P=	0.001	0.001	0.001	0.001		
catalase	r=	0.23	0.34	0.29	-0.23	0.40	-
	P=	NS	NS	NS	NS	0.05	

eight weeks:

		plasma Mg	plasma Cu	Cp	liver wt	liver Cu	Cat
plasma Cu	r=	0.63	-				
	P=	0.001					
Cp	r=	0.60	0.94	-			
	P=	0.001	0.001				
liver wt	r=	-0.70	-0.59	-0.53	-		
	P=	0.001	0.001	0.01			
liver Cu	r=	0.73	0.90	0.82	-0.76	-	
	P=	0.001	0.001	0.001	0.001		
catalase	r=	0.36	0.41	0.53	-0.54	0.44	-
	P=	NS	0.05	0.01	0.01	0.05	

Table 144. Summary of the regression analyses' parameter estimates in Experiment 1 using plasma magnesium as the independent variable at three weeks and eight weeks.

three weeks:

variable	intercept	b_1	PR > T	b_2	PR > T
PHA CPM[a]	3148.81	5167.41	0.7888	-1768.93	0.8083
PHA SI[b]	1.21	14.45	0.6887	-3.78	0.7814
Con A CPM	88480.71	-77441.27	0.6098	32090.29	0.5761
Con A SI	91.59	4.79	0.9864	5.43	0.9591
PWM CPM	42751.74	-46124.99	0.2738	17183.72	0.2805
PWM SI	33.89	4.37	0.9584	-2.20	0.9446
antibody titer	47.30	-61.37	0.7693	40.35	0.6108
log titer	4.52	5.54	0.8134	-0.17	0.9850

eight weeks:

variable	intercept	b_1	PR > T	b_2	PR > T
PHA CPM	16924.89	-17059.34	0.3043	5425.17	0.3071
PHA SI	32.39	-38.92	0.3048	13.34	0.2834
Con A CPM	93895.78	-85369.33	0.2989	22936.59	0.3806
Con A SI	236.04	-285.50	0.0947	89.35	0.1015
PWM CPM	15412.93	-12760.29	0.3095	3210.68	0.4212
PWM SI	32.94	-37.12	0.1267	11.11	0.1513
antibody titer	-159.51	382.44	0.2166	-113.74	0.2491
log titer	0.13	21.13	0.2017	-5.71	0.2772

a, counts per minute
b, stimulation index

Table 145. Summary of the regression analyses' parameter estimates in Experiment 2 using plasma copper as the independent variable at four weeks and eight weeks.

four weeks:

variable	intercept	b_1	PR > T	b_2	PR > T
PHA CPM[a]	164.22	2897.01	0.0298	-1731.64	0.0317
PHA SI[b]	1.40	8.16	0.0515	-5.08	0.0459
Con A CPM	1900.67	15964.00	0.1055	-10560.50	0.0785
Con A SI	10.72	50.37	0.1605	-34.52	0.1142
PWM CPM	1680.93	2827.67	0.2941	-2209.48	0.1791
PWM SI	5.60	10.82	0.2145	-8.03	0.1311
antibody titer	15.36	9.99	0.7372	-6.34	0.7246
log titer	5.61	3.48	0.6629	-2.26	0.6402

eight weeks:

variable	intercept	b_1	PR > T	b_2	PR > T
PHA CPM	237.08	4719.81	0.4912	-2278.57	0.5626
PHA SI	6.04	6.80	0.7708	-2.87	0.8303
Con A CPM	20283.46	-5503.58	0.8512	4065.33	0.8097
Con A SI	138.34	-125.97	0.4275	70.49	0.4399
PWM CPM	1790.34	2815.65	0.5340	-989.26	0.7031
PWM SI	12.73	1.07	0.9509	1.16	0.9074
antibody titer	38.80	-74.87	0.0012	41.85	0.0016
log titer	5.61	3.48	0.6629	-2.26	0.6402

a, counts per minute
b, stimulation index

Table 146. Summary of the regression analyses' parameter estimates in Experiment 3 using plasma magnesium as the independent variable at four weeks and eight weeks.

four weeks:

variable	intercept	b_1	PR > T	b_2	PR > T
antibody titer	52.88	-138.70	0.6932	118.50	0.4000
log titer	4.79	10.27	0.4620	-1.33	0.8099

eight weeks:

variable	intercept	b_1	PR > T	b_2	PR > T
PHA CPM[a]	145.79	2104.56	0.7792	-114.48	0.9725
PHA SI[b]	2.31	6.21	0.7528	-1.83	0.8338
Con A CPM	12854.60	25361.36	0.6114	-9622.54	0.6627
Con A SI	115.17	-6.57	0.9732	-8.99	0.9171
PWM CPM	-2716.36	30952.71	0.2242	-13119.97	0.2436
PWM SI	39.95	13.24	0.8690	-10.33	0.7713
antibody titer	-124.14	347.71	0.1116	-135.88	0.1574
log titer	-17.65	57.65	0.0080	-23.11	0.0150

a, counts per minute
b, stimulation index

Table 147. Summary of the regression analyses' parameter estimates in Experiment 3 using plasma copper as the independent variable at four weeks and eight weeks.

four weeks:

variable	intercept	b_1	PR > T	b_2	PR > T
antibody titer	108.88	-328.36	0.3381	297.37	0.1136
log titer	9.34	2.93	0.8211	4.38	0.5328

eight weeks:

variable	intercept	b_1	PR > T	b_2	PR > T
PHA CPM[a]	76.65	6942.82	0.2028	-3282.15	0.2457
PHA SI[b]	0.85	18.11	0.1950	-9.18	0.2061
Con A CPM	11567.37	38253.76	0.2768	-17046.69	0.3499
Con A SI	67.47	103.80	0.4555	-63.70	0.3797
PWM CPM	2996.05	18890.35	0.2860	-7150.63	0.4349
PWM SI	25.48	29.36	0.6121	-13.74	0.6480
antibody titer	48.77	-21.30	0.8921	33.58	0.6815
log titer	6.90	11.27	0.4943	-3.52	0.6809

a, counts per minute
b, stimulation index

VITA

Marlene Marie Most Windhauser was born in Lincoln, Nebraska on March 20, 1954. She then moved with her family to Denver, Colorado. Throughout her teen years, she was active in the 4-H program. Among her honors were the Outstanding 4-H Member of Colorado, and the 4-H Presidential Award from the U.S. President for the top young woman in the nation in citizenship.

In 1972, Marlene graduated from Arapahoe High School, Littleton, Colorado. She attended Colorado State University where she earned a B.S. in food science and nutrition with a second major in general home economics in December, 1975. She then received a M.S. in consumer sciences with a minor in nutrition in May, 1977.

After completing a six-month work experience at Proctor Community Hospital in Peoria, Illinois, Marlene became a registered dietitian, and worked for the Mississippi Public Health Department. She then worked for the North Mississippi Retardation Center in Oxford as a clinical dietitian. In 1981, she became the food service director.

In 1983, Marlene began her work on a doctorate in the Department of Physiology, Pharmacology, and Toxicology at Louisiana State University, with a minor in nutrition.

Marlene is married to John W. Windhauser, and owns two German Shepards, Greta and Haley.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Marlene Marie Most Windhauser

Major Field: Veterinary Medical Sciences (Physiology Option)

Title of Dissertation: "The Effects of Marginal and Severe Dietary Magnesium and Copper Deficiencies on Immune Function"

Approved:

Leonard C Kappel
Major Professor and Chairman

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Dean of the Graduate School

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December 2, 1988